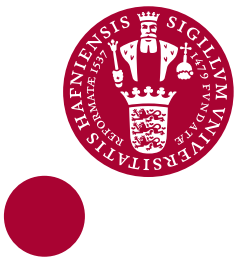

Species identification of skins and development of sheep wool

An interdisciplinary study combining textile research, archaeology, and biomolecular methods

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PhD thesis

Luise Ørsted Brandt

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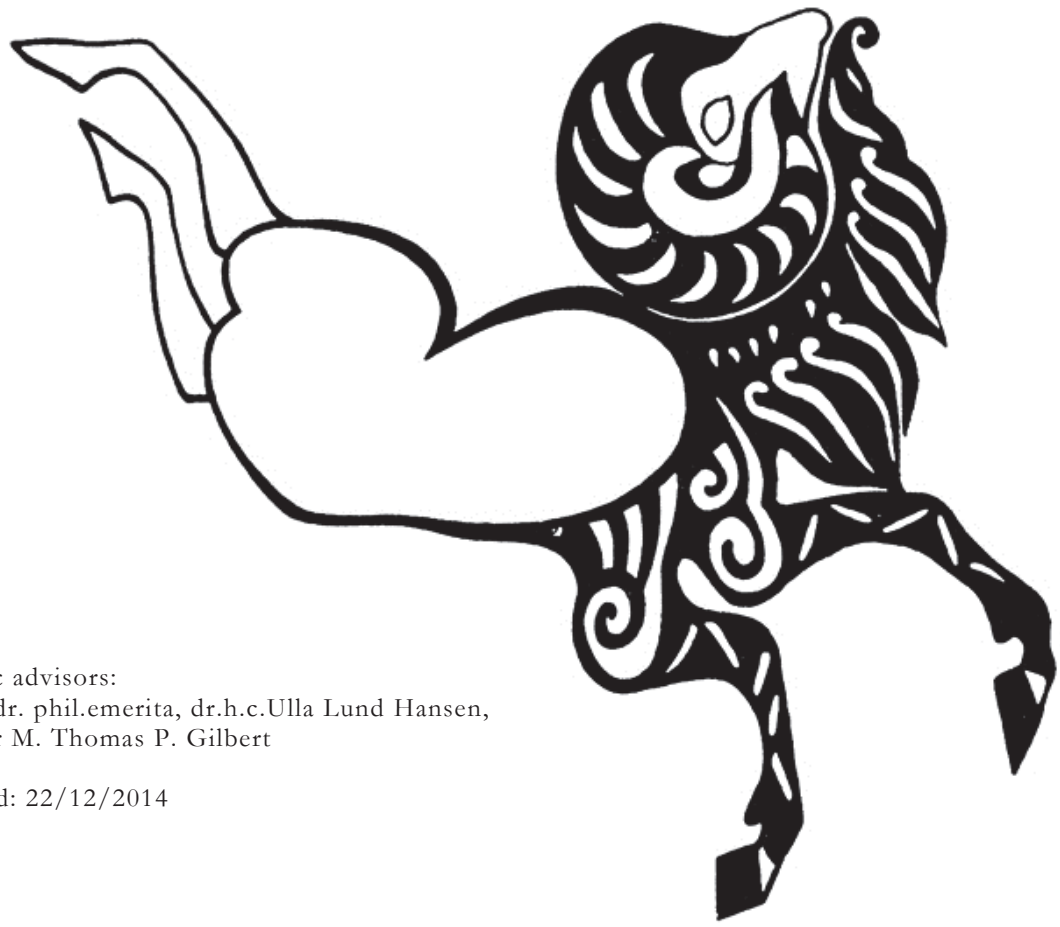

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Professor M. Thomas P. Gilbert

Submitted: 22/12/2014

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Professor M. Thomas P. Gilbert

Submitted: 22/12/2014

To my parents

For their ever support and backing

Preface

This multidisciplinary PhD Thesis was submitted to the Faculty of Humanities, Department of Prehistoric Archaeology, the SAXO Institute, University of Copenhagen. The research was made possible by a grant from the SAXO Institute, University of Copenhagen and The Danish National Research Foundation's Centre for Textile Research (CTR), for which I am very grateful.

The multidisciplinary research was carried out as a collaboration between CTR and The National Research Foundation's Centre for GeoGenetics, at which the laboratory work was conducted.

The title of the advertised PhD grant was *Archaeology, ancient DNA, and textile research* and was part of CTRs TECC program (TExtile Crafts and Cultures) that aims at investigating the development of Scandinavian textile and skin production and costume development until c. AD 400.

Through the three years of the PhD, funding from the Danish Ministry of Culture enabled a study of Danish archaeological skins, which provided me with the opportunity to learn mass spectrometry-based sequencing of ancient peptides. The project has thus led me through a variety of fields including archaeology, zooarchaeology, ancient DNA, and ancient proteins.

The setup of a Danish PhD (three years) allocates two years to a project, half a year to course work and half a year of service commitment to the host institution. During my PhD, I therefore among others had the pleasure of organizing and co-teaching a full course (15 ECTS) in Arctic costumes at the University of Copenhagen and of co-organizing and co-teaching the same course at the University of Greenland. Morever I have taught a laboratory project, *Extraction and sequencing of mammoth DNA*, at the Natural History Museum of Denmark, University of Copenhagen, and been a guest lecturer at the University of Copenhagen. I have also presented my research at various conferences and given outreach presentations as well as written popular scientific articles not included in my PhD. These tasks have, in addition to the project itself, given me the opportunity to develop my skills both as a person and as a researcher and for this I am grateful.

Copenhagen, December 2014

Luise Ørsted Brandt

Acknowledgements

First of all I would like to thank the SAXO Institute, University of Copenhagen and The Danish National Research Foundations Center for Textile Research for giving me the opportunity to write this PhD Thesis and the Ministry of Culture for their funding of the species identification project included in this Thesis. I would also like to thank Center for GeoGenetics for kindly allowing me to use their laboratories.

I thank my supervisors docent dr. phil.emerita, dr. h.c. Ulla Lund Hansen, the SAXO Institute, and Professor M. T. P. Gilbert, Center for GeoGenetics for their indispensable guidance and professional discussions. I also thank Enrico Cappellini for his help and guidance in mass spectrometry which was a completely new field to me.

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I thank all my excellent and friendly collegues at both Center for Textile Research and Center for GeoGenetics for their invaluable help and for creating a great working environment. A special thank to my friend and soon to be dr. Cecilie Brøns for many weekends at the Danish National Museum, her always available cheering, and discussions of PhD life in general.

Last but not least I would like to thank my friends and family for encouragements and cheering.

Abstract

Denmark possesses an extraordinary collection of well-preserved textiles and skins from Danish prehistory as well as archaeological and historical skins and costumes from the circumpolar area. Much research has focused on how these textiles and skin garments were produced. Recently, textile research has endeavoured to move beyond this and investigate prehistoric costumes as the output of interactions between resources, technology and society. As some of these aspects are difficult to investigate archaeologically, especially studies of the character of the raw materials used for textile and skin production, fresh approaches are needed, including new methods.

This thesis investigates archaeological and historical skin garments and textiles using an interdisciplinary approach that combines biomolecular methods, archaeology and textile research. The aims of this thesis are first to investigate the development of sheep wool and secondly to species identify archaeological and historical skins.

Sheep wool development is investigated by archaeological material, state-of-the-art textile research, and next generation sequencing (NGS) of ancient sheep DNA. Archaeological sources point to the development of a sheep wool that could be used for textile production in the Danish LN II or EBA I (2000-1500 BC). Changes of the wool seem to again take place in the Roman Iron Age (AD 1-400).

The genetic analysis of DNA from wool textiles and sheep bones aimed at extracting the entire mitogenome and nuclear markers coding for wool quality. None of the included textiles yielded amplifiable DNA, confirming that environments preserving ancient Danish textiles are generally poor for the preservation of DNA. Two bone samples yielded almost complete mitogenomes, while only a very few of the targeted nuclear markers mapped to the reference genome. The resolution of sheep mitochondrial DNA is, however, too low to generate detailed information of sheep population history and therefore nuclear DNA should be the focus in the future.

Three studies focused on the species identification of skins. Traditional species identification by microscopy was proven to be difficult to perform here on ancient and degraded hair materials. Therefore, additional approaches are needed. For those samples not deriving from highly degrading environments, such as the analyzed skins of historical costumes from the circumpolar area, traditional PCR is still a valuable tool for species identification. For samples from degrading enviroments, such as the Danish bogs, Mass Spectrometry (MS)-based peptide sequencing was proven to be a reliable method for species identification. Moreover, MS-based peptide sequencing provided information of the age at death for one of the animal skin samples - information not obtainable by DNA and with crucial implications for the interpretations of preferences of skins and animal husbandry.

Online available protein databases used for comparison are still not complete. While the most common domesticated species are well described, the databases did not provide enough resolution of seals and birds to presently justify the species identification by PMF of ancient Greenlandic skin samples dating to the Saqqaq culture.

Overall, the success of the analysis of ancient biomolecules is closely connected to the nature and preservation conditions of the sample, and the ability to answer archaeological questions depends on the questions posed and the methods chosen. When such questions are considered, biomolecular methods can provide a good tool to characterize ancient raw materials and investigate their interwoven relationships with production contexts and society.

Resumé på dansk

Den danske muld har leveret en uovertruffen samling af velbevarede tekstiler og skind fra den danske forhistorie. Derudover er Danmark i besiddelse af en stor samling af arkæologiske og historiske skind og dragter fra det cirkumpolare område. Meget forskning har fokuseret på hvordan disse tekstiler og skinddragter blev produceret. På det seneste har tekstilforskningen dog også forsøgt at studere de samspil mellem resourcer, teknologi og samfund som ligger bag de forhistoriske dragter. Fordi nogle af disse aspekter er svære at udforske med traditionelle arkæologiske metoder, herunder karakteren af de råmaterialer som blev brugt til fremstillingen af dragter af skind og tekstil, er nye fremgangsmåder nødvendige, inklusiv nye metoder.

Denne afhandling undersøger arkæologiske og historiske skinddragter og tekstiler med en tværfaglig fremgangsmåde, der kombinerer biomolekylære metoder med arkæologi og tekstilforskning. Afhandlingens formål er for det første at undersøge udviklingen af fåreulden og for det andet at artsbestemme arkæologiske og historiske skind.

Fåreuldens udvikling undersøges udfra arkæologiske kilder, den nyeste tekstilforskning og next generation sequencing (NGS) af gammelt fåre DNA.

De arkæologiske kilder peger på, at en uld, der kunne anvendes til tekstilproduktion blev udviklet i SNII eller ÆBA I (2000-1500 f.Kr.) Ændringer i ulden ser igen ud til at finde sted i romersk jernalder (1-400 e.Kr.).

De genetiske analyser af DNA fra uldtekstiler og fåreknogler sigtede mod at udvinde det fulde mitokondrielle genom og markører af kerne DNA, som koder for uldens kvalitet. Det var ikke muligt at udvinde DNA fra nogle af de inkluderede tekstiler, hvilket bekræfter, at DNA bevares dårligt i de miljøer, der er optimale for bevarelsen af forhistoriske tekstiler.

Et næsten fuldt mitokondrielt genom kunne udvindes fra to af de inkluderede knogleprøver, mens kun få udvundne sekvenser matchede de tilsigtede kerne DNA markører. Opløsningen af mitokondrie DNA hos får er dog for lav til at frembringe detaljeret information om fårets populationshistorie og derfor må fokus lægges på udvindelsen af kerne DNA i fremtiden.

Tre studier i afhandlingen fokuserede på artsbestemmelsen af skind. Den traditionelle artsbestemmelse ved mikroskopi blev her vist at være vanskelig at udføre på forhistoriske og nedbrudte hår. Derfor er supplerende metoder nødvendige. For prøver der ikke stammer fra miljøer der er nedbrydende for DNA, som de inkluderede skind fra historiske dragter fra det cirkumpolare område, er traditionel PCR stadig en værdifuld metode til artsbestemmelse. For prøver der kommer fra miljøer der nedbryder DNA, som fx de danske moser, viste Mass Spectrometry (MS)-based peptide sequencing sig som en sikker metode til artsbestemmelse. Derudover gav MS-based peptide sequencing information om alderen på et af de analyserede skind – en information, som ikke kan opnås ved analysen af DNA og med vigtige implikationer for fortolkningen af skind præferencer og husdyrhold.

Offentligt tilgængelige databaser over proteiner, som bruges til sammenligning er endnu ikke komplette. Mens de mest almindelige tamdyr er velbeskrevne, gav databaserne ikke nok opløsning for sæler og fugle til, på det nuværende tidspunkt, at artsbestemmelse ved PMF på prøverne fra de grønlandske skind, dateret til Saqqaq kulturen, kunne retfærdiggøres.

Samlet set er succesen af analyser af forhistoriske biomolekyler tæt forbundet med karakteren og bevaringen af en prøve, og muligheden for at besvare arkæologiske spørgsmål afhænger af de stillede spørgsmål og den valgte metode. Når dette overvejes kan biomolekylære metoder udgøre et godt redskab til at karakterisere forhistoriske råmaterialer og undersøge forholdet mellem disse, produktion og samfund.

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PART I

Introduction

"Naar Archæologen har behandlet sit Stof efter dets Art og Beskaffenhed, efter Fundomstændigheder og Forhold til andet beslægtet Stof og under sammenstilling med Alt, hvad der fra nære eller fjærne Omraader, fra ældre eller senere Tider maatte foreligge til Oplysning, vil den egentlig faglig archaæologiske Behandling være endt. Men ofte kan Undersøgelsen ikke siges hermed at være fuldt afsluttet. Hvad der er formet eller tilvirket I Menneskets Haand, henhører med Hensyn til selve Fabrikationsmaaden under Techniken og Haandværket; forsaavidt der foreligger naturhistoriske Levninger, ville disse behøve Naturforskernes Bestemmelse, og strengt taget kræve alle egentlige Oldsager en videnskabelig Undersøgelse med Hensyn til Stoffet, hvoraf de bestaa. Ved den redebonne Bistand, der ikke mindst I de senere Aar er blevet ydet til en saadan videre Undersøgelse af det her I Landet fremdragne archæologiske Materiale, fra Zoologiens, Geologiens, Botanikkens og Anthropologiens Side, er der blevet modtaget mange værdifulde Oplysninger."
Bille Gram 1891 (Gram 1891: 97).

When the Archaeologist has treated his material by its kind and nature, by the circumstances of its find and the relationship to other related material and in comparison with all, that from near or faraway areas, from early or later times, might lay for enlightenment, will the real scientific archaeological treatment have ended.
But often the investigation cannot hereby be said to be fully completed. What has been shaped or manufactured in the hands of man belongs, in relation to the way of manufacturing, to Technology and Trade; so far as there are natural historical remains, these would need identification of the natural historians, and strictly speaking all actual antiquities require a scientific investigation with regards to the material of which they consist.
By the ready assistance, which not least in later years have been provided to such a further investigation of the, here in the country, unearthed archaeological material, by zoology, geology, botany and anthropology, there has been received much valuable information.[1]

[1] Translation by Louise Ludvigsen, The Danish National Research Foundations Center for Textile Research

Introduction to the thesis

Archaeological skin and textiles accommodated basic human needs as clothing and shelter. Textiles and skins were moreover used for a variety of purposes including carrying and wrapping objects, for furnishing, as sails, and to signal identity via for instance flags or specific clothing. They were fundamental and their production was timeconsuming, involving workforce, raw materials and technologies. Due to its importance, archaeological textiles have been shown to represent a significant economy in several ancient societies. Textiles and skin garments were produced by humans, but they also constrained and enabled human activities. This relationship between cloth and humans makes archaeological skins and textiles an excellent source to study essential processes og aspects of human life (Barber 1991, Good 2001, Andersson Strand et al. 2010).

Textiles and skin garments are rare in archaeological contexts. Because of their organic nature, they are rapidly decomposed after disposal. Nevertheless, cloth is preserved under extremely advantageous circumstances including waterlogged, frozen, saline or very dry environments. Moreover textiles can be preserved by exposure to fire, which carbonizes them or by contact with metals that mineralizes them (Andersson Strand et al. 2010: 151-152).

Denmark possesses an extraordinary collection of well-preserved textiles and skins from Danish prehistory as well as archaeological and historical skins and costumes from the circumpolar area. This remarkable collection was the starting point of this dissertation.

Historically, textile research has focused on how textiles and skin clothes were produced and of what raw material. For textiles such analysis includes its dimensions, the yarn and the fibres, and its structure, construction and decoration (Andersson Strand et al. 2010: 153). During recent decades, textile research has attempted to move beyond this, and use prehistoric costumes to investigate the society behind them. New analyses view textiles as the output of interactions between resources, technology and society (Andersson Strand et al. 2010: 150). Some aspects of these intertwined relationships are particularly difficult to investigate archaeologically, including studies of the character of the raw materials used for textile and skin production. Therefore new approaches are needed, including new methods.

This thesis investigates archaeological and historical skin and textiles using an interdisciplinary approach which combines biomolecular methods with theory and methods from archaeology.

Aims

The aims of this interdisciplinary investigation of archaeological and historical skin and textiles are:

1. Species identification of archaeological and historical skins from Denmark and the Arctic.

Identification of animal species is an important part of studies of skin as it provides information of available animal species, animal exploitation and choice of skin for different purposes. Traditional species identification by microscopy was shown to be difficult on ancient samples (Schmidt et al. 2011). Therefore a species identification method based in a secure scientific foundation is needed to provide preferably more trustworthy characterisations of the skins.

2. Investigation of the development of sheep wool.

The development from the coat of wild sheep to a sheep wool type that could be used for textile production and its development over time has been studied extensively. Previous research includes studies of sheep bone assemblages, textile tools, written sources, wool fibre development etc. When and how wool developed is, however, still unclear. The development of sheep wool had an immense impact on humans as it enabled a new way of dressing and initiated new technologies. To provide new information on sheep and sheep wool development, state of the art archaeology and textile research is here combined with ancient DNA analysis.

Background for reseach of prehistoric costume in Denmark

Excellent preservation conditions have provided Denmark with an outstanding collection of prehistoric wool textiles and skins. The Danish textiles and skins come from three main contexts: oak log coffins, bogs and inhumation graves. The earliest textiles are dated to around period I of the Early Bronze Age (1700-1500 BC). The earliest textiles were preserved in the waterlogged conditions of oak coffins embedded in burial mounds. A large group of textiles and skins dating to the Iron Age (500 BC-800 AD) were preserved in peat bogs, where they were deposited, occasionally along with a body. The majority of these were found during peat cutting in the 20th century. The third group of textiles derive from inhumation graves dating from the Bronze, Iron Age and Viking Period (1700 BC-1050AD) which had particularly benefitial environments for preservation of organic material.

Many of the Danish prehistoric textiles were found in the 19th and 20th century during peat cutting, farming activities and construction work (Mannering et al. 2012: 91). Initially, the textiles were mainly published as part of finds of human bodies (see for instance the bog body from Haraldskær (Anon. Oldsags-commiteen 1836-37) and the oak coffin grave from Egtved (Thomsen 1929). It was not until Hans Christian Broholm and Margrethe Hald published the Bronze Age textiles in 1935 (Broholm and Hald 1935, 1940) that a collected presentation of Danish textiles with comparisons was published. This work was followed up in 1950 by Hald's publication of the Danish Iron Age textiles and costumes (Hald 1950, 1980). These works laid the initial ground for Danish textile research.

In 1974 Elisabeth Munksgaard published a book on Danish prehistoric costumes, that represented many of the well-known Danish textile finds. This work became an extensively used overview of Danish dressing through time (Munksgaaard 1974). One of the most remarkable books in Danish textile research was published in 1986. Lise Bender Jørgensen's publication of Prehistoric Scandinavian Textiles included 95% of the Danish textile finds that were known at that time (Bender Jørgensen 1986). Bender Jørgensen's publication was also innovative, in the way that it introduced analyses of weaving techniques, cloth types, spinning directions and thread count. This work and her subsequent publication of North European Textiles in 1992 (Bender Jørgensen 1992) have been the foundation for much of the modern textile research carried out in Denmark and Northern Europe.

Since 2005, the Danish National Research Foundation's Centre for Textile Research (CTR) has contributed significantly to the research of historic and prehistoric textiles and their societies, both in Denmark and internationally. The CTR has demonstrated that textile research must not only examine

the textiles themselves, but also explore the connection between textile, environment, and society. Apart from fundamental research of Mediterranean and Danish textiles (Andersson Strand and Nosch in press, Mannering and Gleba in prep.), CTR has launched initiatives and collaborated with research groups in crossdisciplinary fields as textile terminologies (Michel and Nosch 2010) and epigraphy and iconography (Nosch et al. 2013), and have widened their area to also include studies of textiles from India and China (Nosch et al. in press). The work on the further development of existing methods and the application of new scientific methods to textile research have been a major priority for the CTR. These methods include dye analysis to identify dyes and mordants (Berghe et al. 2009, 2010), strontium isotope analysis to trace the provenance of ancient textiles (Frei et al. 2009a, Frei et al. 2009b), fibre analysis to investigate processing of wool (Rast-Eicher 2008, Skals in prep.), tool studies (Andersson Strand 2008, 2010, 2012) and DNA analysis to characterise prehistoric sheep (Brandt et al. 2011a). These new approaches have already yielded exciting new results and will hopefully continue to do so.

Arctic costumes and archaeological skins

Due to the Danish Realm, the Danish collection of skins and textiles also include historical costumes from Greenland and the circumpolar area. Many of these were collected by missionaries, government officers and explorers in the 19th and 20th centuries and have thus never been buried. The costumes have been the focus of a large-scale strategic project *Northern Worlds* at the National Museum of Denmark (Schmidt 2010, 2012) and some of these are analysed in chapter 3.

The National Museum of Denmark moreover has close collaborations with the Greenland National Museum. Many archaeological excavations in Greenland have been carried out by Danish researchers. The cold environment of Greenland provides excellent conditions for the preservation of organic materials as wood, textiles, and skins. This is also the case for the Saqqaq settlement Qeqertasussuk from which skin samples (dating to around 2000 BC) analysed in chapter 4 derives. The material from Qeqertasussuk is currently being published by Bjarne Grønnow, of SILA, at the National Museum of Denmark.

Geographical frame

As mentioned above, Denmark possesses an extraordinary collection of prehistoric costumes recovered from Danish bogs and burials. Though in some regards peripheral to developments in Europe and the Middle East, this area may provide one of the best options to study the development of prehistoric textiles and costumes, as preservation conditions have left far fewer garments closer to the proposed

centre of textile and fibre development. The geographical focus for CTR's TECC program (TExtile Crafts and Cultures), is therefore naturally Scandinavia. Danish, Norwegian and Swedish textiles provide an opportunity to study Scandinavian prehistoric textile and costume development, but are also highly relevant to the discussion of costume development in Central Europe and the Middle East.

The material in chapter 1 and 2 was therefore limited to Danish finds in order to be able to cover several time periods, contexts, and regions, within the time frame of the study - though bordering, historically and culturally related areas could be relevant to include.

Chronological frame

Skin garments deposited in Danish bogs are most frequent in the period from 500BC-400AD (Mannering et al. 2010: 266). The skin capes, of which some are included in chapter 2, date from the transition from the Bronze Age to the Iron age around 500 BC to the end of the Migration Period AD 550[2] (Ebbesen 2009: 42, Mannering et al. 2010: 264-65). This period is therefore the focus for the analysis of proteins from Danish skin capes.

As sheep bones are not found prior to around 4000 BC in Denmark, this time point is a natural beginning for studying the development of sheep and wool fibres. The time focus of CTR's TECC program is the time period until AD 400, during which developments in sheep wool are supposed to have taken place. However, I also include samples from Germanic Iron Age, Viking Period and the Medieval Period, in order to compare with samples from periods when fibres and textile production were even more developed and perhaps specialised. The chronology of Danish time periods used is shown below and refers to works of various scholars as listed in the following table.

Period name	**Absolute dating**	**Chronology after**
Early Neolithic	3950-3300 BC	Nielsen 1993: 85
Middle Neolithic	3300-2400 BC	Nielsen 1993: 85
Late Neolithic	2400-1700 BC	Nielsen 1993: 85
Early Bronze Age	1700-1100 BC	Montelius 1885, Randsborg 1996, Vandkilde et al. 1996, Olsen et al. 2011
Late Bronze Age	1100-500 BC	Montelius 1885, Randsborg 1996, Vandkilde et al. 1996, Olsen et al. 2011
Pre-Roman Iron Age	500-1 BC	Becker 1961: 268-71
Early Roman Iron Age	AD 1-150/160	Lund Hansen 1995: 18
Late Roman Iron Age	AD 200-400	Lund Hansen 1995: 18
Early Germanic Iron Age	AD 400-520/540	Jørgensen and Jørgensen 1997: 38
Late Germanic Iron Age	AD 520/540-800	Jørgensen and Jørgensen 1997: 38
Viking Period	AD 800-1050	Roesdahl 2001: 14-15

Table 1. Applied chronologies and absolute datings.

[2] However, two of the finds, Hørby Mose and Årestrup By (number 4 and 6 in the catalog in Ebbesen 2009) are undated.

Structure of the thesis

This thesis comprises three parts. Part I introduces the background, theory and methods of the thesis, Part II which makes up the core of the thesis comprises four studies in which biomolecular methods are applied to archaeological and historic costumes. The first study of Part II explores when the fleece of sheep changed from the original coat of wild sheep to a fleece that could be used for textile production. It includes an investigation based on evidence from archaeology and textile research and an ancient DNA analysis of Danish sheep. The aim is to convert into an article for submission to the Journal of Archaeological Science. The three last studies focus on species identification. The second study identifies species that constitute skins from Danish Iron Age capes found in peat bogs, and aims at developing a reliable and reproduceable method for species idenfication of skins from peat bogs. This study also includes an article published in PLoS ONE (Brandt et al. 2014). The third study aims at species identification of skins in costumes from the circumpolar area and the fourth study aims at species identification of skin fragments from the Greenlandic Saqqaq settlement of Qeqertasussuk. Part III closes the thesis and concludes on the studies and proposes future directions of research.

Theory

Working in both two scientific fields, archaeology and biology, it has become clear to me that researchers in these fields have very different approaches to the production of knowledge and its truth value. This chapter aims to establish an approach for navigating in the fields of biology, archaeology and the cross-disciplinary field of archaeological science. This is done by demonstrating how the production and conception of knowledge differ between research traditions of the humanities and the natural sciences.

This theoretical approach is established by clarifying how the concept of theory is understood and leads to the production of knowledge in biology and archaeology respectively. By introducing the field of archaeological science or archaeometry, it is discussed how studies that combine different research traditions can be carried out and how scientific results can contribute to and be incorporated into an analytical framework in archaeology are discussed. As the overall purpose of this dissertation is to arrive at a meaningful way of combining scientific data and archaeological research, the aim of this chapter is not to review interdisciplinarity theory but to create such a meaningful approach through selected central works.

Production of knowledge in the natural and social sciences and the term 'theory'

Studies of ancient biomolecules (see definition in next paragraph) build on theories and methods from both biology and archaeology. These two research traditions are very similar, as their knowledge is empirical, i.e. based on observations. Both traditions advance hypotheses for material based on these observations, and investigate whether the consequences of a hypothesis fits the material. Thus, a hypothesis can be rejected or accepted. However, the definition of theory and the conception of knowledge, the epistemology, vary within the two research traditions.

In biology, a scientific theory can be defined, as for example by The American Association for the Advancement of Science as: …*a well-substantiated explanation of some aspect of the natural world, based on a body of facts that have been repeatedly confirmed through observation and experiment. Such fact-supported theories are not "guesses" but reliable accounts of the real world.* [3]

As this quote explicitly demonstrates, natural science aims to describe the natural world, and natural scientists believe that they can objectively produce facts about a true natural world.

The above quote is an expression of the theoretical position of objectivism, which is perceived as an essential theoretical basis for the natural sciences (Jones 2004: 4). Objectivism views the natural world as being made up of objects that can be studied accurately by human subjects viewing them externally (Jones 2004: 4). The prerequisite for such statements is the belief that the natural world is static. Only by acknowledging that the natural world is constant and unchangeable, can the scientist provide a valid, consistent description of it and its objects (Jones 2004: 4). The relationship between objects is viewed as causal, meaning that a certain cause has a certain effect on an object, which I think is well illustrated in the drawing by Jacques de Vaucanson, demonstrating the digestion of a duck (Fig. 1). Investigation and repeated confirmation of such causal chains enable natural scientists to put forward laws that explain the relationship between cause and effect (causality) and predict outcomes of a situation. These are used for deductive reasoning, which is widespread in biology. Knowledge in biology is thus produced from tested theories and facts and is considered to mirror the truth about the natural world.

[3] http://www.aaas.org/news/press_room/evolution/qanda.shtml .

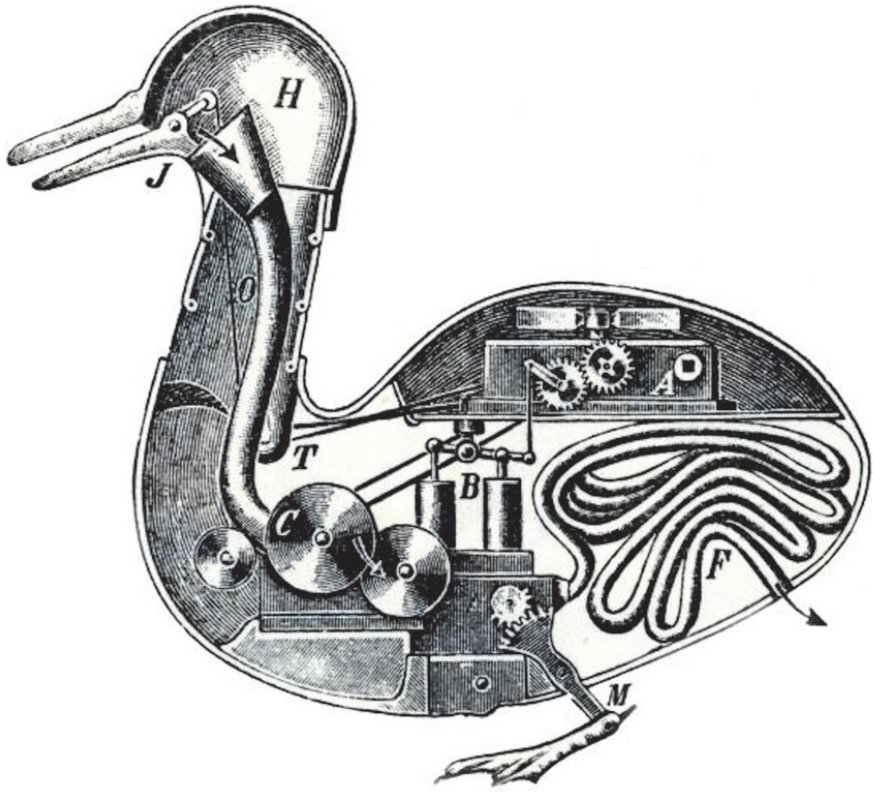

Figure 1. The digestion of a duck. Drawing by Jacques de Vaucanson demonstrating natural scientific causality.

The field of social sciences differs fundamentally from the natural sciences as its purpose is to describe society and not the natural world.

Archaeology has experienced different theoretical paradigms with different ways of perceiving and producing knowledge. The most recent paradigms are presented below. The processual paradigm, was launched by Lewis Binford's publication of *Archaeology as anthropology* in 1962 in which he defined culture as: *"…the extrasomatic means of adaption for the human organism"* (Binford 1962: 22) and stated that: *"...change in the total cultural system must be viewed in an adaptive context both social and environmental..."* (Binford 1962: 20).

Binford's idea was that, culture is a tool for ecological adaption, and that it was, therefore, possible to compare cultures with equal ecological environments as they would react equally to equal terms. This

enabled comparisons between societies, including ethnographic analogies, from which knowledge of ancient societies could be produced (for instance Binford 1978).

The processualist paradigm dominated primarily in the period from the 1960s to the 1980s, until it was challenged in the 1980s by, amongst others, Ian Hodder. Hodder stated that: *"... culture is not man´s extrasomatic means of adaption but that it is meaningfully constituted"* (Hodder 1982: 13) and that *"Man´s actions and his intelligent adaption must be understood as historically and contextually specific, and the uniqueness of cultural forms must be explained"* (Hodder 1982: 13). According to Hodder and the post-processual paradigm, culture is historically and culturally specific and only makes sense in its own cultural context. Post-processualist archaeologists believe that the archaeological record is not made of inanimate objects but of material remains bearing encoded information about the past. It is then the task of the archaeologist to translate this code or read the past (Hodder 1986, Tilley 1991). Due to the distance in time and culture between the archaeological culture studied and the archaeologist, such a reading of the past will always be conditioned by the cultural background of the archaeologist (Shanks and Tilley 1987: 105-106). According to the post-processualist paradigm, knowledge is, therefore, never "true", but based on interpretations, and is constantly subject to change (Olsen 1997: 94).

In contrast to the definition of theory advanced by The American Association for the Advancement of Science, in archaeology, theory in social science does not equal a hypothesis or a model as in the natural sciences. In my understanding, theory is a critical approach to interpreting data, drawing attention to the assumptions and historical backgrounds underlying, for instance previous interpretations or models, and asking critical questions to the material. Following this definition, it often makes more sense to speak of theoretical perspectives or aspects when dealing with archaeological theory.

As demonstrated above, the natural sciences aim at studying the natural world, whereas the social sciences study society. Natural scientists view the world as something that can be studied objectively and produce "facts" about this world, whereas social scientists study a changing society and recognise that they are unable to produce "true" data. Studying ancient biomolecules thus unites research traditions that comprise very different theoretical approaches. Incorporating "facts" from natural scientific analysis into archaeological post-processual analysis will, however, bring archaeology back to objectivism.

I will return to how scientific data can be incorporated into the field of archaeology after an introduction to the field of biomolecular archaeology and its research history.

The field of archaeological science and biomolecular archaeology

Biomolecular archaeology is defined as the study of ancient biomolecules in order to address archaeological questions (Brown and Brown 2011: 289). The biomolecules studied in biomolecular archaeology are the organic macromolecules found in living organisms in a, more or less, degraded state after their death: nucleic acids (deoxyribonucleic acid, DNA and ribonucleic acid, RNA), proteins, lipids, and carbohydrates (Brown and Brown 2011: 4).

Studies of ancient biomolecules fall within the field of archaeological science, also known as archaeometry. To the best of my knowledge, there is no fixed definition of these terms. However, both archaeological science and archaeometry cover the cross-disciplinary studies, in which methods from the natural sciences are applied to archaeological material to address archaeological problems and questions. The term archaeological science has been criticised for indicating that archaeology is not a science in itself (Ehrenreich 1995). Though I consider archaeology a science I will, however, apply this term, as it to me directs the attention to the diversity of auxiliary sciences applied in archaeology, and not just the ones that include measures as archaeometry points to.

As demonstrated by the starting quote used in the introduction, the use of natural scientific methods has a long history in Danish archaeology (Gram 1891: 97). Archaeological sciences have the ability to contribute to archaeology by addressing archaeological questions that cannot be investigated by traditional archaeological methods. Such questions range from the dating of artefacts and archaeological sites to the composition of archaeological materials, to more complex questions, such as the development of humans and their cultures (McGovern et al. 1995).

Early archaeological scientific methods included archaeobotany, palynology, zooarchaeology, dendrochronology and human osteology. The dating of artefacts and studies of their composition, technological production and provenence are also widely applied. Along with methodological developments in the natural sciences, the number of methods applied in archaeology have increased rapidly and also fields, such as isotopic analysis and studies of ancient biomolecules have become very popular (see review of science in archaeology in McGovern et al. 1995). Proteins and lipids were the first biomolecules studies from archaeological contexts (Boyd and Boyd 1934, Boyd and Boyd 1937, Condamin et al. 1976), nevertheless, DNA yields by far the most studies on ancient biomolecules today. The focus of the present dissertation is DNA and proteins which are introduced in more detail in the following paragraphs.

Research history of biomolecular archaeological studies

Until the 1980s, DNA-based genetic analyses were restricted to the high quality DNA found in modern tissues. In 1984, Higuchi et al. published a study in which DNA techniques were succesfully applied to an extinct member of the horse family, the quagga (Higuchi et al. 1984: 282). This first study was quickly followed up by a study of ancient DNA in an Egyptian mummy (Pääbo 1985: 645). Although given the failure of subsequent studies to replicate the findings using samples from a similar context, many now believe this latter example to have been derived from contaminant modern DNA.

These early studies showed that DNA could survive in ancient tissues even though only short and damaged fragments of multi-copy loci (genes present in many copies), such as mitochondrial DNA, could be extracted. Due to DNA damage (see method section), retrieval of DNA from ancient materials was highly complicated (Willerslev and Cooper 2005: 3). This problem was partly overcome when PCR (Polymerase Chain Reaction) was taken into use (Saiki et al. 1988), as PCR in theory is capable of producing millions of copies from a single fragment of DNA (see method section, Willerslev and Cooper 2005: 3). The great amplifying power of PCR, however, also increased the risk of contamination from modern DNA. Consequently, many of the studies that followed Higuchi et al. 1984 and Pääbo et al. 1985 have turned out to be false positive (Willerslev and Cooper 2005: 3). Therefore, in 2000 Cooper and Poinar published 10 criteria for authenticity (Cooper and Poinar 2000). These were followed up in 2005 by Gilbert et al. who stated, that applying the criteria blindly would not always be sensible. (Gilbert et al. 2005). The increasing knowledge about contamination, degradation and survival of DNA has made aDNA research an important field of research, and today, the method is applied to many different tissues (Willerslev and Cooper 2005: 5).

Proteins were the starting point of biomolecular archaeology and the research into them goes back to blood typing of Egyptian and Native American mummies with immunological methods in the 1930s. However, the typing methods turned out to be unspecific, working on ancient and degraded samples (Brown and Brown 2011: 38). With the developments of proteomic techniques over the last few years and advances in knowledge on the degradation of proteins, research into ancient proteins (paleoproeomics) has become more significant in biomolecular archaeology (Brown and Brown 2011: 39-40).

As demonstrated, analysis in ancient DNA and proteins can characterise ancient materials, but how can such data be incorporated into an archaeological analysis? This forms the topic of the next section.

Incorporation of scientific results in archaeology

Science has been percieved as more capable of providing truth and "facts" than arts (Jones 2004: 2). There are several objections to such a perception. My main objections deal first with the perception of the ability to produce "facts" about the past, and secondly with the value attributed to these "facts".

To start with the validity of scientific data, the perception of these as "true" is highly controversial – at least, within the humanities. The validity of scientific data has been addressed by the French sociologist and anthropologist Bruno Latour (1947-). Latour's authorship counts numerous works that cover a wide range of themes (Blok and Jensen 2009). Central for this dissertation is especially his famous anthropological study conducted in a laboratory in the USA in collaboration with the British sociologist Steve Woolgar, in which they explored how facts are constructed in the natural sciences (Latour and Woolgar 1979, 1986). Latour and Woolgar's observation of laboratory scientists led them to several eye-opening conclusions. Particularly interesting for the discussion of the scientific production of facts, was Latour and Woolgar's challenging of the perception of viewing scientific facts as "truth". They state that a large part of scientific training involves learning how to make the subjective decision of what data to keep and what data to reject (Latour and Woolgar 1979, 1986). This highlights that humans are active in the production of scientific data and that human sorting mechanisms and decision making affect the conclusions and the production of knowledge. Following this train of thought that knowledge is constructed by humans, then it must be constructed differently in different cultural contexts and times. Scientific results are, therefore, not objective, but formed by ruling paradigms and the techniques and theoretical background available at a certain time. The extreme position of such thinking is the notion that all laws put forward by the natural sciences must be questioned constantly and that no real natural world exists to be described, but only culturally contingent interpretations of it.

Trained in the post-processual paradigm, I do not believe in the production of true knowledge. However, as an archaeological scientist, I do believe that the accurately defined practices and reproducibility of scientific methods have the strength to provide characterisations of archaeological material, which is definitely different from providing evidence about past societies. In this study, I therefore accept the scientific data that I have critically produced based on the selected material, the selected method, the data-analysis after specific parameters and incomplete databases. I also accept that this data is only contingent based on the present cultural context, which is based on our present knowledge.

The second objection presented regards the value of "fact". One may argue that, facts do not make sense in themselves, and that they do not contribute to the project of archaeology and the humanities, which is exploring what it means to be human in the present and past, without an interpretation. How this can be carried out will be explored in more detail below.

In this thesis, the concept of materiality is applied to navigate both natural science and archaeology. In contrast to material culture, which comprises the tangible objects we surround ourselves with, materiality can be defined as the relation between these objects and humans (Kragelund and Otto 2005: 5-13). In this relationship, objects are constructed by humans, but also constrain and enable human activities (Jones 2004: 330). This influence of objects upon humans has been termed material agency.
The acknowledgement that objects can possess material agency emphasises that their properties and quality have an effect on their perception and use, and that they shape human behaviour (Jones 2004: 330). The properties of material culture studied by archaeological science studies can thus have an important effect on human behaviour. However, the properties of objects will be perceived according to their cultural context. Through the concept of materiality, scientific data (according to the above critical approach) can be incorporated into post-processual frameworks of analysis (Jones 2004: 332), providing information on material properties whose effect on human society can be explored.
In the next paragraph some of the main conflicts between archaeologists and scientists is discussed. As apparent in the below paragraphs, one of the core disputes is that of representativity which will also be dealt with in the following.

Reflections on bio-archaeological studies

Since its beginning, the number of studies of ancient biomolecules have increased dramatically (Hofreiter et al. 2001a). Many of these studies were performed by scientists with a background in biology or genetics, and although the material was paleontological or archaeological, the analyses were performed within a natural scientific paradigm aiming at investigating evolutionary processes, past population dynamics, the evolutionary relationship between extant and extinct species and past ecosystems. However, several studies also aim at answering archaeological questions. Some of these studies aim at providing specific information about one or a few samples, as for instance studies of the ice mummy Ötzi and his clothing (Hollemeyer et al. 2008, 2012, Olivieri et al. 2012). Other studies aim at answering more complicated and speculative questions, such as human migrations, kinship and population dynamics. Although both types, in my opinion, operate within a natural scientific paradigm,

the latter type has been hugely criticized by archaeologists for, amongst others, its material representativity and generalisations.

An example of this is an ancient DNA study that was criticized by archaeologists. The study was published in 2010 by Rasmussen et al. and investigates the peopling of the Arctic. In the study Rasmussen et al. sequence the entire genome of a 4000-year-old Greenlander, and provide genetic evidence that the individual sampled was closely related to the present-day population of the Aleutians – a group of islands stretching from Siberia into the Pacific Ocean (Rasmussen et al. 2010). This result was published as evidence that the entire Saqqaq population descended from a migration from Siberia into the New World. The study was subsequently highly criticized by researchers from SILA – The Arctic Research Centre at the Ethnographic Collections, National Museum of Denmark (Rasmussen et al. 2010). The critique pointed partly at the exclusion of humanities researchers in the interpretation of the results, the exclusion of prior archaeological research on the question, and the context of the sample. Generally, archaeological critique points first at the representativity of the studies. Often, only a single or a few samples which may be widely spaced in time, are used to draw general conclusions on a much larger material or answer complex archaeological questions. Secondly, the archaeological context of the samples may not be considered, even though it yields valuable information on the sample and the questions that can be used to analyse it. Thirdly, archaeologists are often offended by the complete exclusion of decades and even hundreds of years of archaeological research, which also provides evidence for the analysis of archaeological questions.

In my opinion, the theoretical gap between archaeology and biology led to a reluctance amongst archaeologists to apply genetic methods. However, several large-scale projects[4], including researchers with backgrounds from biology, the humanities and other disciplines, have lately been launched to bridge the two sciences. This has occurred concurrently with technological developments and a drop in prices of analysis. However, such projects still face the challenge of constructing projects that ask questions that take the available archaeological material, its potentials, the available methods and possible questions into consideration. This requires a large degree of collaboration and knowledge sharing between the scientific branches.

Reflections on material representativity

As discussed in the previous paragraphs, the results and conclusions of a study are based on the method and material chosen. Therefore, apart from the method, the material size and its proposed

[4] The ERC-funded project, The Rise (http://the-rise.se/) and the Genomic History of Denmark (http://forskning.ku.dk/styrkeomraader/stjerneprogrammer/genomic_history_of_denmark/).

representativity are central for the conclusions of the study, and whether these conclusions can be generalised and extrapolated to a larger material.

Often researchers aim at stating something general, even if this can only be done on the basis of a small amount of material (by induction) which is not representative i.e. represent the entirety. Yet, only by including the entire material can the analysis be valid for the caracteristics investigated. A random sample will always entail uncertainty which is reduced if the random sample is increased.
In archaeology, even including the entire material recovered will not make up a representative analysis, as several factors determine if artefacts are preserved or not. The extant find material is, therefore, never representative for the once existing material. The processes affecting artefact preservation is investigated in the next paragraph.

Taphonomy

The term, taphonomy was invented by the Russian palaeontologist I. A. Efremov (1940) from the Greek words *taphos* (burial) and *nomos* (law). By this constructed term, Efremov aimed to characterize the transition process of organic material, from the biosphere into the lithosphere, meaning the processes involved when former living organisms turn into fossils. The term taphonomy is normally applied to bone assemblages but the ideas of processes that only determine the partial survival of archaeological material can be applied to other material groups as well.

Working with bone assemblages, the excavated and registred bone assemblage very rarely represents the original death assemblage (Lyman 2008, O'Connor 2004, Noe-Nygaard 1987, Wigh 2001: 33f, Fig. 2). Several factors can affect the size and composition of the original death assemblage and therefore affect the representativity of the material. Such factors are called taphonomic and may be divided into two categories: biostratinomy and diagenesis. The first covers the factors that work on a bone assemblage from the death to its final burial. These factors include, amongst many others, climate, weathering, predation, human marrow fracturing and toolmaking (Noe-Nygaard 1987, Lymann 2008). The second, diagenesis, covers the factors working on the material during its burial to the collection stage, such as character of the sediment, its pH and the access to water. Therefore, preservation conditions provide highly pertinent information for obtaining an idea of the original assemblage. Both biostratinomic and diagenetic factors can bias the presence of bones, skeletal parts or even species in the bone assemblage. Studies that fail to consider taphonomic factors pontentially base conclusions on

a biased dataset, which will affect the result. Therefore, the possiblity for taphonomic factors working on a bone assemblage should be investigated so that this may be taken into account.

Representativity is a general problem in archaeology. Some topics, materials and time periods are harder to investigate than others. Organic materials, such as textiles and skins require exceptional preservation conditions in order to survive in archaeological environments. As already mentioned in the introduction, many textiles and skins are preserved in burial contexts. However, as also mentioned, the instances in which these were preserved are rare, and in some time periods, remains of textiles and skins are almost non existing. This can be due to, for instance burial customs as in the Late Bronze Age and Early Iron Age where cremation graves dominate. Performing a representative analysis in archaeology is, therefore, impossible. In cases where the material is clearly unevenly preserved, other artefact groups can be included to cover the gaps. As in the case of textiles and skins, tools for their production can provide information of their presence and the technologies used to produce them.

To summarize: the theoretical approach applied in this dissertation is interpretive, offering critically produced scientific data of artefact properties incorporated in an post-prosessual archaeological analysis. Due to the character of archaeological material and the framework of a Danish PhD (see preface) the analysis often covers only a few samples spaced over large time spans. The representativity of the results is, therefore, an issue that is considered under each study. However, this does not only apply to the present thesis specifically, but constitutes the premise for carrying out all archaeological studies.

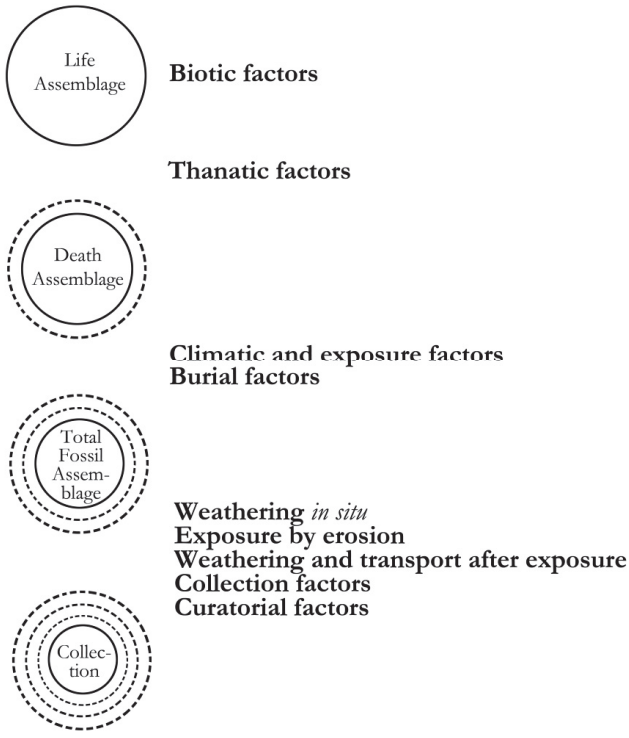

Figure 2. Taphonomic processes effecting the size and character of an archaeological material. Modified after Noe-Nygaard 1987: 8.

Methods: ancient DNA and proteins

Ancient DNA (aDNA) – background and methods

This chapter introduces DNA as a molecule and describes its nature in archaeological materials and some of the methods by which it can be studied as demonstrated in part II. Initially its chemical structure and degradation patterns are described.

The chemical structure of DNA

DNA (deoxyribonucleic acid) is the hereditary material of all known living organisms. The genetic information of the double stranded DNA molecule is stored as a 'code' made up of a sequence of four chemical bases: adenine (A), thymine (T), cytosine (C), and guanine (G). These bases pair with each other, so that A always pairs with T and C always pairs with G. The pairs are often referred to as base pairs (bp). Each base is attached to a sugar and a phosphate molecule, and these three combined make a nucleotide. Nucleotides are arranged in two long strands that form a spiral shape, called the double helix structure. The strands are linked together by the bases, and the sugar and phosphate molecules form the sides of the spiral (Fig. 3). The genetic information stored in the DNA strand is used by the cell to produce proteins that maintain the cell's function through strictly regulated processes. All three nucleotides code for an amino acid, which are linked together to proteins through protein biosynthesis.

Under advantageous conditions DNA can survive in archaeological materials such as bone, hair, nail, and skin, as well as in soil, plant materials and coprolites. This means that the genetic characteristics of ancient organisms can be studied from their DNA.

Ancient DNA (aDNA) is however generally only present in low amounts and suffers from being highly fragmented and prone to contamination and *post mortem* modifications (Lindahl 1993, Willerslev and Cooper 2005). These characteristics define both the possibilities and limitations of aDNA studies and will be addressed with in the following paragraph.

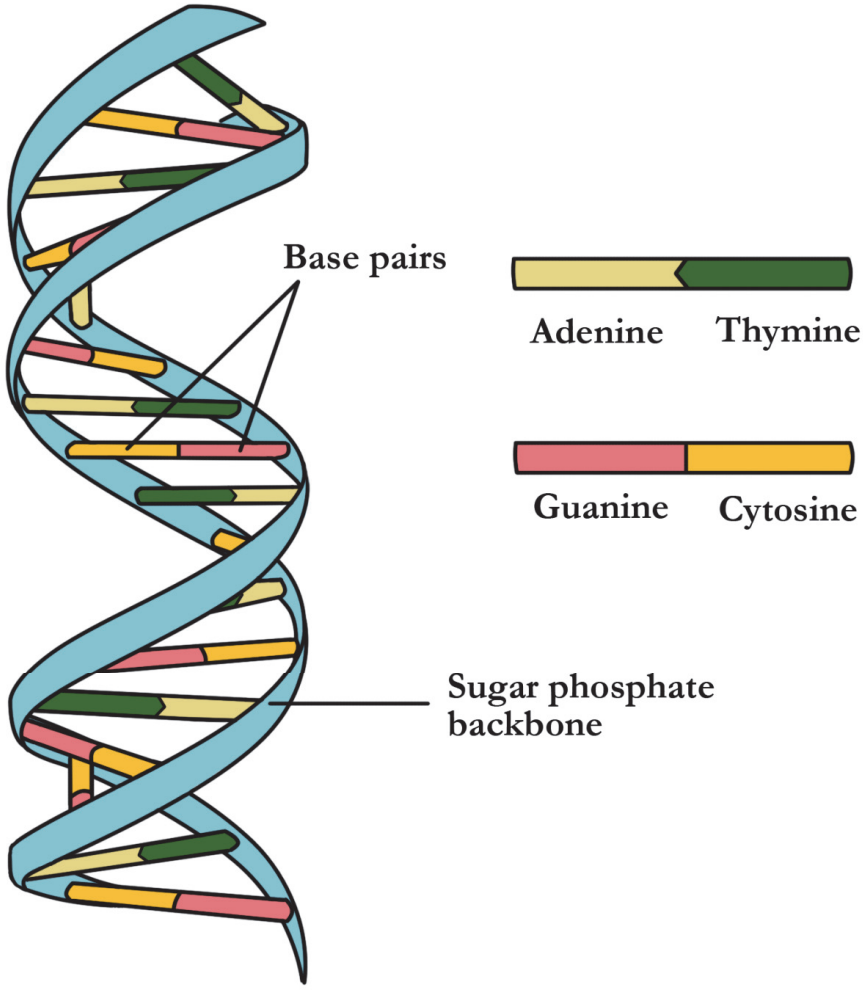

Figure 3. The DNA double helix. The DNA code is made up by the combination of four chemical bases: adenine (A), thymine (T), cytosine (C), and guanine (G) of which A always pair with T and C always pair with G.

Preservation and degradation of ancient biomolecules

In living tissue, DNA damage is rapidy repaired by repair mechanisms (Lindahl 1993). This is, however, not the case for aDNA. Degradation of biomolecules is caused by several factors that can be grouped in three main groups: autolysis, environmental factors, and microbial attacks.

Autolysis is the digestion of biomolecules immediately after an organism's death by endogenous enzymes. In life, these enzymes turn over cellular biomolecules, exogenous molecules and invading bacteria, while the cell´s endogenous molecules are protected by the cell membrane. As the membrane breaks down after death, the cell's biomolecules are exposed to the enzymes and broken down – a process that can be stopped only by exceptional circumstances such as rapid dessication of the tissue, freezing or high concentrations of salt (Brown and Brown 2011: 116). In such cases, other factors as the environment and microorganisms can also modify the biomolecules.

Environmental factors effecting DNA preservation can be of both chemical and physical nature. Among the chemical factors is the presence of water or oxygen, that will cause respectively hydrolytic and oxidative reactions. Under reaction with water (hydrolysis), the DNA strands can break either by cleaving the chemical bonds that bind the nucleotide bases to the sugar-phosphate backbone (depurination), resulting in baseless sites, or cleaving the bonds of the backbone (Lindahl 1993, Willerslev and Cooper 2005). Moreover, hydrolysis can cause base changes (deamidation) which will result in incorporation of eroneous bases during amplification (Willerslev and Cooper 2005). Oxidative damage of the nucleotide bases and the sugar-phosphate backbone prevents amplification and is termed blocking lessions (Lindahl 1993, Willerslev and Cooper 2005). Apart from water and oxygen, pH values that differ significantly from neutral and saline concentrations influence biomolecule preservation (Lindahl 1993, Hofreiter et al. 2001a). Physical factors effecting DNA preservation include radiation and temperature. The last will increase the rate of the reactions caused by all the other factors roughly threefold per 10°C increase (Lindahl and Nyberg 1972, Lindahl 1993), which makes temperature the key factor for biomolecule preservation and degradation (Smith et al. 2001). Temperature is therefore the main factor determining the time it would take DNA to be completely broken down, and the age of a sample that we should expect to be able to recover DNA from (Lindahl 1993, Willerslev, Hansen and Poinar 2004, see Fig. 4). Based on this, cold and dry environments are expected to yield the best conditions for DNA survival, which has been confirmed by several studies (Kumar et al. 2000, Smith et al. 2001, Willerslev et al. 2004).

Lastly, the presence of microbes that prey on biomolecules by secreting enzymes that break them down is an environmental factor important for DNA preservation.

The described DNA damage types make analyses of ancient DNA complicated. For instance the risk of base changes should be taken into account. In addition the breaks in the strand(s) explain the short length of amplifiable products and DNA loss from archaelogical materials (Willerslev and Cooper 2005). The low amounts af endogenous DNA makes ancient samples highly prone to contamination, which can result in false positive results. It is therefore a crucial item of discussion and will be dealt with in the next paragraph.

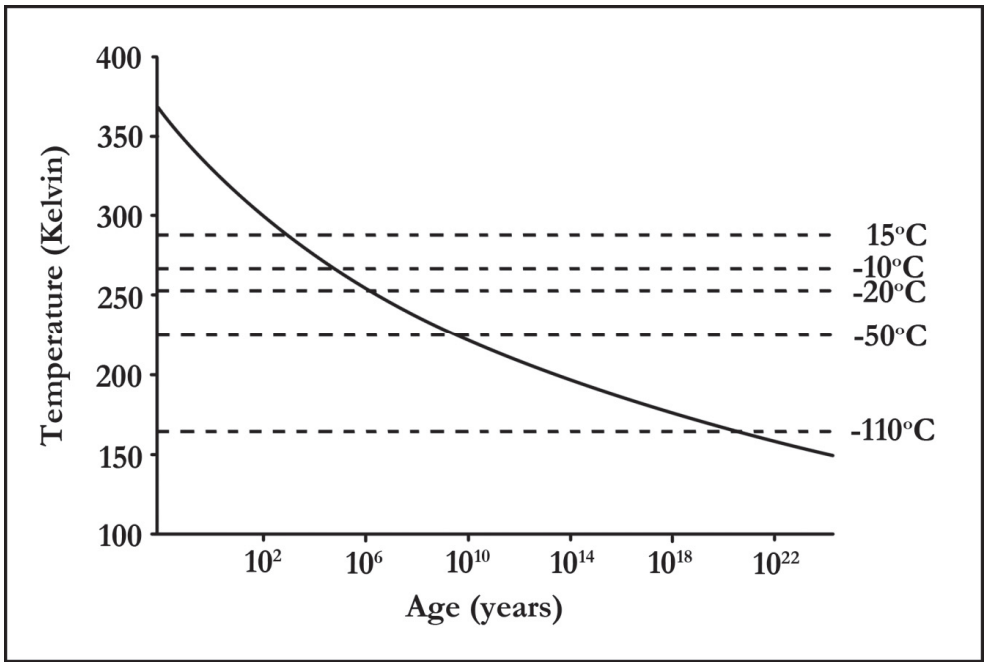

Figure 4. The correlation between temperature and DNA preservation. After Willerslev, Hansen and Poinar 2004.

Contamination

Key issues in ancient DNA research are the risk of contamination and the need to authenticate results (Cooper and Poinar 2000, Gilbert et al. 2005, Hebsgaard, Phillips and Willerslev 2005: 212). By contamination we understand DNA exogenous to the sample. Because aDNA as we have seen is generally present in low amounts and in a degraded state, ancient samples are extremely vulnerable to even minute amounts of exogenous DNA (Willerslev and Cooper 2005: 6), which can quickly exceed the amounts of endogenous DNA in the sample. This problem is exacerbated after PCR amplification which may copy contaminants into the DNA in high amounts (Hebsgaard, Phillips and Willerslev 2005: 212).

Contamination can derive from both modern and ancient sources and occur in several phases of the analysis both before or after arrival to the lab. Careless handling and the use of contaminated reagents and plasticware are common sources of contaminants. However, also general lab contaminations such as aerosols from previous samples may contaminate samples. Contamination can also occur due to cross contamination with other samples. All such issues will of course lead to false results and erroneous interpretations.

The problem that is most difficult to ascertain is if the sample has been contaminated during sampling. This is however less of a problem when working with sheep DNA which is easy to distinguish from human DNA (Hebsgaard, Phillips and Willerslev 2005).

To overcome such issues several precautions and strategies to avoid contamination have been implemented, which is the subject of the next paragraph.

Authenticity

In 2000, after several previous false postitive studies, Cooper and Poinar published a list of criteria of authenticity to follow in order to verify ancient DNA studies and avoid contamination (Cooper and Poinar 2000). The criteria encouraged scientists to work with ancient DNA in conditions physically isolated from modern DNA and PCR (Fig. 5), to use extraction and PCR blanks, replicate data and to conduct an ”appropriate molecular behavior” meaning applying a critical approach to results that seem to good to be true. The initial criteria were, however, followed up by Gilbert et al. in 2005 stating that following these criteria should not lull scientics into false security (Gilbert et al. 2005). They encouraged scientists not to use the criteria as a checklist, but instead to ask themselves if their results were believable.

After this introduction to DNA and its challenges, I shall turn to the methods of the studies included in this dissertation.

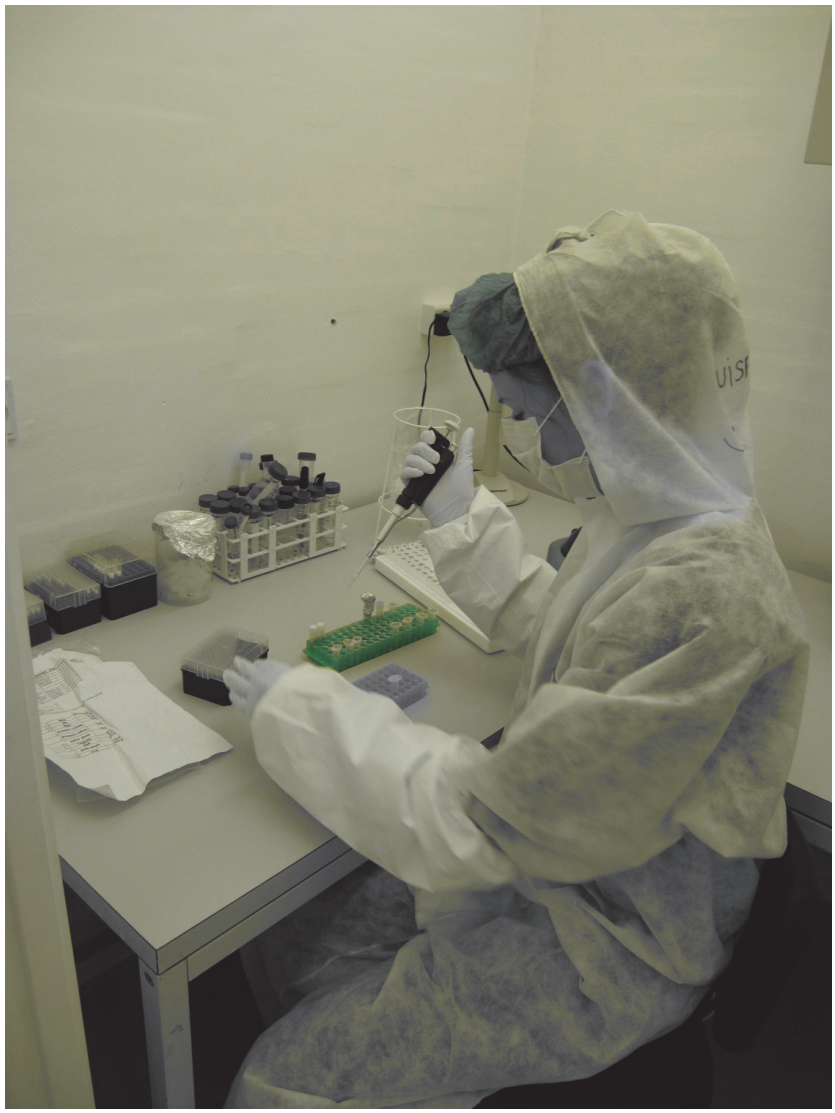

Figure 5. Full bodysuits are worn in the laboratory to prevent contamination.

Methods and development in aDNA research

The field of aDNA has experienced massive changes and improvements from the first studies in the 1980s (see theory section). Early studies only had the possibility to extract and amplify of short fragments of DNA and mitochondrial DNA (mtDNA) was intensively studied. There are several reasons why mtDNA is highly useful for studies of aDNA.

The majority of our DNA is located in the cell nucleus (nuclear DNA); however, only a minority is found in the cell's energy producing mitochondria (mitochondrial DNA or mtDNA). Nuclear DNA is only present in one copy per cell, whereas one cell can contain thousands of copies of mitochondrial DNA (Fig. 6). Because of the numeric difference between these types of DNA, mitochondrial DNA is generally easier to obtain than nuclear DNA - especially if the samples are ancient and degraded. Nevertheless, nuclear DNA holds by far the most information with regards to functionally important genetic traits. Therefore nuclear DNA is more interesting for studies of specific genetic traits.

As mtDNA is only inherited through the maternal lineage, it only changes when mutations occur. Based on these sequence variations, an individual or an archaeological sample can be placed into mitochondrial haplogroups with shared polymorphisms (variable sites) indicating a close relationship in contrast to individuals with differing polymorphisms that will be less closely related. Studies of mtDNA haplogroups have made mtDNA highly valuable as an indicator of maternal kinship and in population genetics and phylogenetic studies (Bruford et al. 2003).

The most common target within the mitochondria has been the hypervariable regions (Fig. 7). These regions are situated in the part of the mitochondrial genome that does not code for anything. As the name indicates, these regions display a high number of polymorphisms (variable sites) (Brown and Brown 2011: 24-25). This means that the hypervariable regions are ideal for studies of population level differentiation, as there will be a good chance to find differences between individuals. However, on the species level the regions vary too much within a single species to be useful as a barcode for distinguishing between species. In these cases more conservative markers as 16S, 12S, and cytochrome b are classic as they seem to vary between species, but not within a species. Thus these markers have been used intensively in species identification studies.

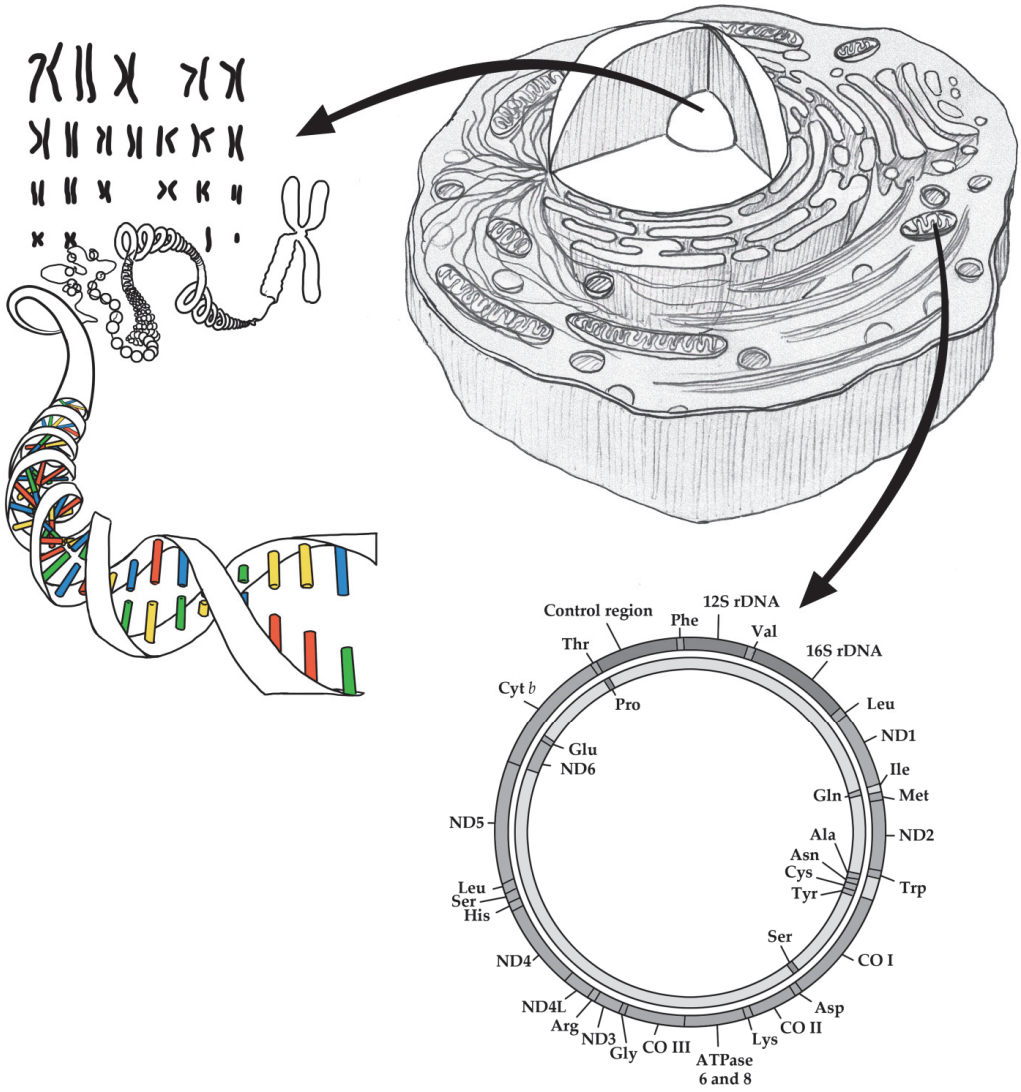

Figure 6. Nuclear and mitochondrial DNA

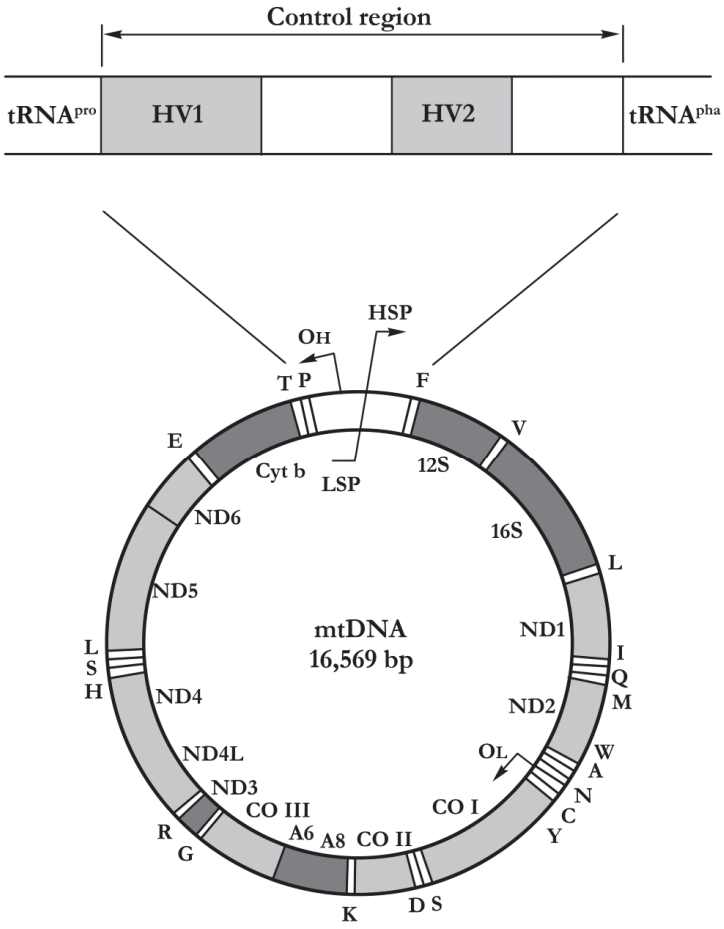

Figure 7. The location of 16S, Cytochrome b and the control region in the mitogenome.

Unlike mitochondrial DNA, nuclear DNA is inherited from both parents. Thus the genome of any offspring is a mix of DNA from both father and mother. Nuclear DNA provides a record of inherited DNA and kinship can be inferred by comparison between the DNA of individuals.

Genes can exist in two or more forms (alleles), which differ by variations in their nucleotide sequence (genotype). Alleles result in significant characteristics of a trait (phenotype) as for instance hair or eye colour, sex, susceptibility to genetic diseases etc. (Hartl and Clark 2007: 5-8). Brown and Brown 2011: 22). The simplest variations are SNPs, Single Nucleotide Polymorphisms, which are cases where just a single nucleotide base differs. In some cases, SNPs cause a change of amino acid during protein biosynthesis, whereas in other cases they do not. In cases where it does, an SNP can make up an allele.

Some genetic traits are more complex than others. There are two types of inheritance of phenotypic traits (Sørensen et al. 2005: 166): qualitative inheritance and quantitative inheritance. Qualitative inheritance is the inheritance of phenotypic traits that are under control of major genes and vary discontinuously in a population. An example of this is blood type, which is influenced by only one gene and has only four possible outcomes: A, B, O, and AB, which are present in the population in very different amounts (in Denmark, A: 44%, B: 10%, O: 41% and AB 5%[5]). Quantitative inheritance on the contrary is the inheritance of phenotypic traits that vary in degree depending upon the cumulative action of many genes and their environment. Therefore it is also referred to as polygenetic inheritance. Quantitative traits vary continuously in a population. An example of this is human height, which is normal distributed, and wool quality, which we will turn back to in chapter 1.

After this introduction to DNA, aDNA, and its properties, we will turn to the methods by which aDNA can be studied.
The workflow in aDNA analysis begins with the extraction and purification of DNA from the ancient specimen. After this, pure DNA can be analysed in very different ways. Tradionally, it was amplified by Polymerase Chain Reaction (PCR), but techniques have evolved rapidly over the past decade and Next Generation Sequencing (NGS) techniques have taken over the field. In the following, the workflow of PCR and NGS will be explained.

Methods within ancient DNA

Since shortly after its introduction in 1988 (Saiki et al. 1988) and until NGS took off within the last decade, PCR has been the predominant method to amplify ancient DNA (Metzker 2010). The power of PCR consists in its ability to amplify low copy numbers of ancient DNA sequences into quantities that can be sequenced (Brown and Brown 2011: 26). In the PCR reaction, amongst others, an enzyme, polymerase, nucleotides and primers (a target specific sequence that will bind to the DNA strand on each side of the target) is added to the DNA extract (Fig. 8). PCR amplification is performed in a machine that for each cycle doubles the amount of DNA. A cycle contains three temperature steps: denaturation, annealing, and elongation. The first, at around 95°C, causes the double stranded DNA molecule to fall apart. During annealing, the primers attach to each of the strands under a temperature determined by the composition of the primers. Finally, during elongation at 72°C, the enzyme inserts

[5] http://da.wikipedia.org/wiki/Blodtype

nucleotides into the single stranded molecules, making new double stranded molecules. If successful, the products may be cloned and Sanger-sequenced (Brown and Brown 2011: 24; Sanger et al. 1977). PCR has yielded an enormous amount of studies on ancient samples. Because of the nature of ancient samples, sequences of more than 200 bp are normally hard to retrieve using PCR (Poinar et al. 2006). Moreover PCR targets specific sequences of DNA determined by the primer set used. Also, short sequences are hard to retrieve as the amplicons at least have to cover two primers of about 20 base pairs. These issues entail limitations for the use of PCR on ancient samples.

PCR and Sanger sequencing remains in wide use, primarily for smaller-scale projects, as demonstrated in chapter 3. However, since the first study applying pyrosequencing (Margulies et al. 2005) NGS has taken over much of the aDNA studies carried out and has changed the field by making large-scale genome analyses and working on short sequences possible (Metzker 2010).

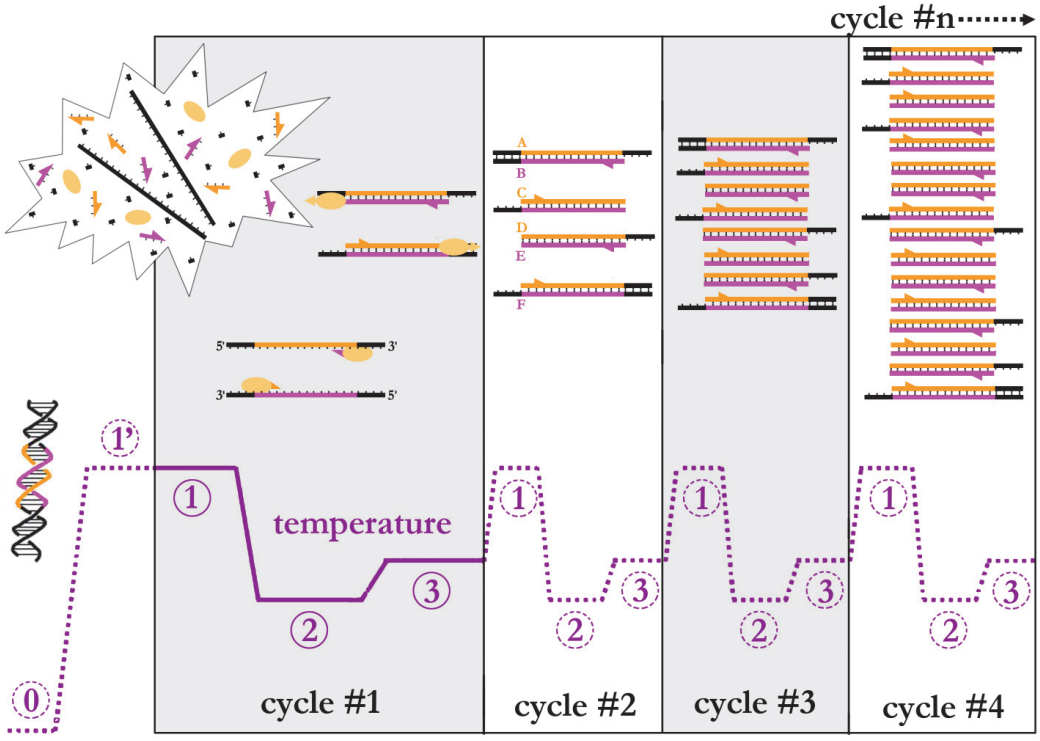

Figure 8. The principle of the PCR. Enzyme, nucleotides and primers are added to the DNA extract. During each cycle, the double stranded DNA is denatured and the enzyme incorporates nucleotides at the target forming to double stranded molecules. Each cycle doubles the amount of DNA in the reaction.

Illumina library construction

Next generation sequencing techniques generally involves the building of so-called libraries as demonstrated in chapter 1 (Metzker 2010). Libraries are DNA sequences from a sample that are provided with a tag (also referred to as a barcode or an index) that allows pooling and sequencing of multiple samples.

The building of libraries is divided into several steps (Meyer and Kircher 2010, see Fig. 9).

First, overhanging ends of both strands are enzymatically blunted to create a double stranded molecule of equal length (Fig. 9, 1). Then adaptors are attached to the blunt end of one strand by the enzyme ligase (Fig. 9, 2). The nicks (strand discontinuities) created thereby are removed with a reaction that fills in the gaps (Fig. 9, 3). The adaptors are used as priming sites for the subsequent amplification of the library. As mentioned above, the primers used contain a tag that is made up of a short sequence (for instance 6 bp) unique to each library in the analysis (Fig. 9, 4). Indexed libraries can be sequenced directly using platforms such as the Illumina MiSeq or HiSeq series, or enriched prior to sequencing using target-capture methodologies.

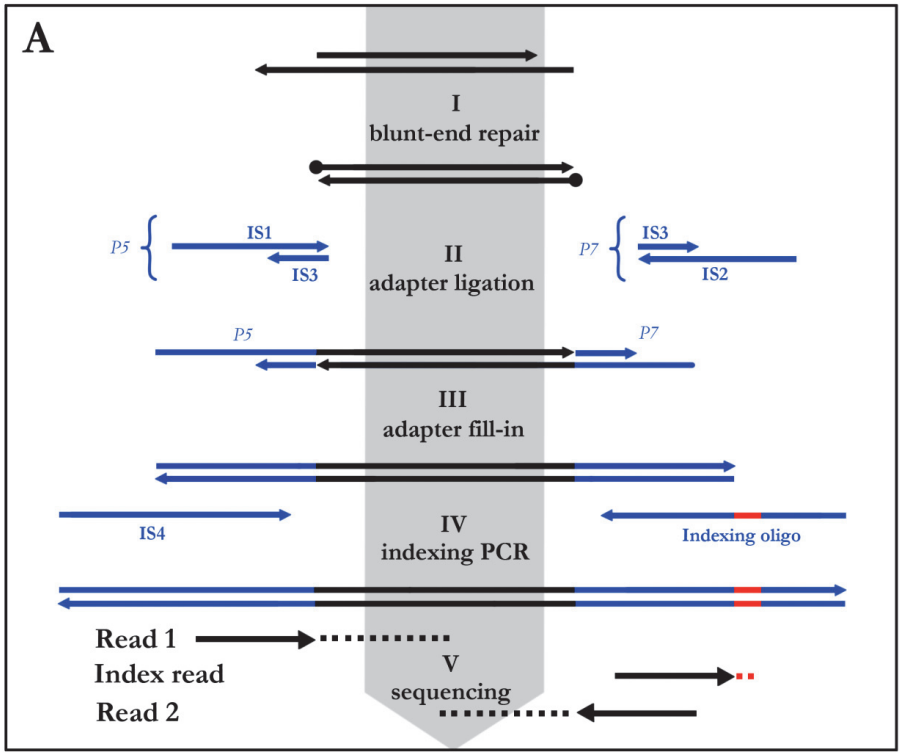

Figure 9. Preparation of libraries.
1. Blunt ending. 2. Adaptor ligation. 3. Adaptor fill-in. 4. Indexing PCR.

Target-capture-sequencing

Target-capture-sequencing (Gnirke et al. 2009) is a NGS approach that can increase the cost efficiency of aDNA studies. The method is a more advanced method than the traditional PCR applied in the previous studies by Brandt (Brandt et al. 2011a, 2011b) and enables sequencing of even extremely short fragments (under 50 bp) of both the entire mitochondrial and nuclear DNA. This means that archaeological materials deriving from environments where DNA is degraded to very short fragments could yield amplifiable DNA with target-capture-sequencing, whereas traditional PCR would fail to yield DNA. Also, this new method allows for the amplification and sequencing of multiple sequences at a time. Timewise, it would be impossible to amplify this number of sequences using traditional PCR. Target-capture-sequencing is a clear improvement compared to the method used in the first studies of textiles by Brandt et al. (2011), in which only a few and short sequences of mitochondrial DNA was extracted and sequenced.

The method makes use of the combination of predesigned bait and libraries to extract and enrich specific targets in the genome.

In the approach demonstrated by Maricic et al. (2010), bait is produced through the generation of biotinylated PCR products targetting the regions of interest in the target species (in chapter 1 both the complete mitogenome and SNPs). If long range PCR products are used, which is the case if the mitogenome is the target, these are fragmented. If ancient DNA is the target, long range PCRs are fragmented to under 500 bp as only short products are suspected to bind.

Adaptors are then ligated to the bait and by denaturation, the bait is made singlestranded. The adaptors are used to immobilise the bait on biotinylated beads. The doublestranded libraries (see earlier) are also denatured and singlestranded library molecules are captured by the bait on the beads (Fig. 10, bottom). The method this way captures many targeted molecules in one process, whereas regions of less interest will not be sequenced. Subsequently, the captured molecules are eluted from the bait, amplified and sequenced.

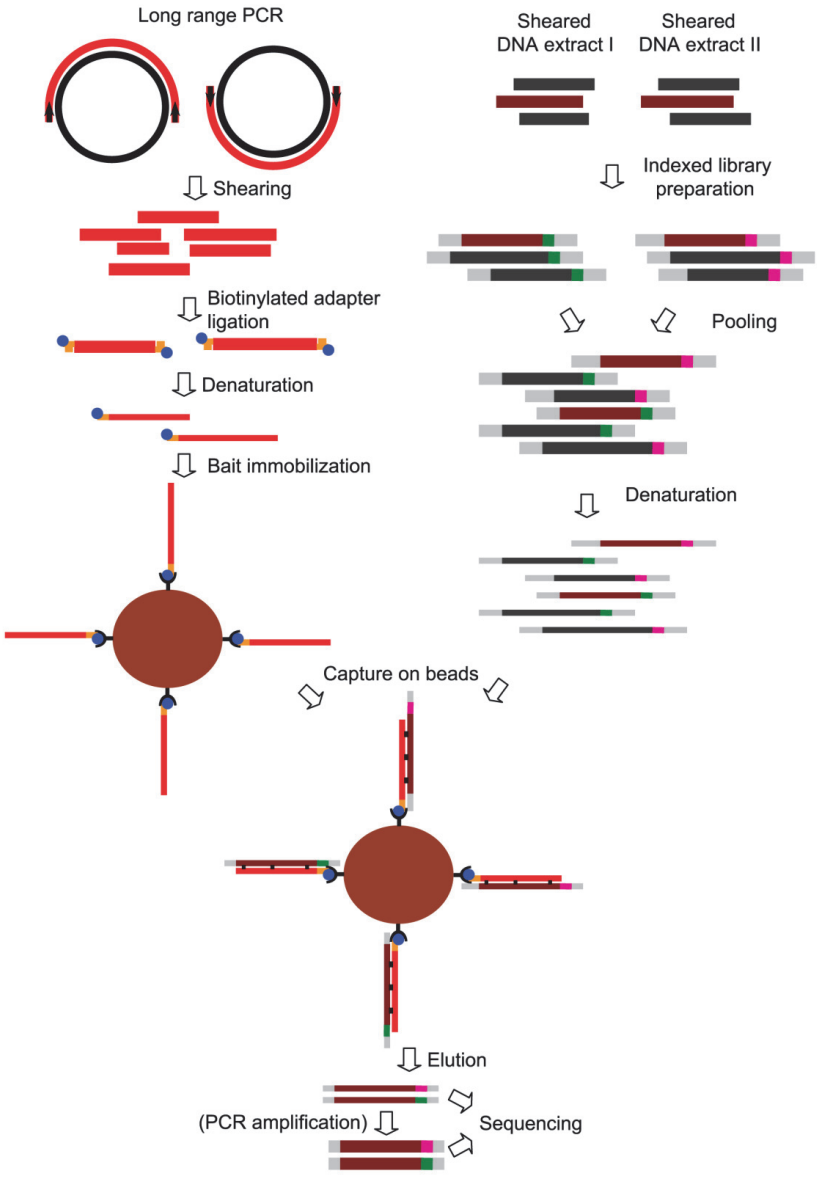

Figure 10.
Left: the production of the immobilized bait from long range PCR products.
Right: the production pooled indexed libraries is displayed.
Bottom: the bait and pooled libraries are used in the capture. The eluted molecules are first amplified and then sequenced. Light red: bait. Dark red: mitochondrial DNA in the libraries. Green and pink: indices. Grey: adapters. Thicker lines: double stranded DNA. Thinner lines: single stranded DNA. After Maricic et al. 2010.

In the context of my research, the use of this approach enables (in theory) the study of genetic diversity within sheep in greater depth, and to look for changes in specific genes over time. In this dissertation, genes coding for wool quality are of particular interest.

With well-preserved DNA it is therefore possible to investigate kinship and functional traits as sex and coat colour (see for instance Bouwman 2008, Melchior et al. 2008, 2010, Svensson et al. 2012). However, DNA analysis is still restricted:

"The range of characteristics that can be addressed is limited, because molecular biologists have only an incomplete understanding of the link between DNA structure and biological attributes, and for many characteristics the link is complex and not easily unraveled by examining the DNA."

Brown and Brown 2011: 10

Most genetic data derived from ancient individuals is analysed in comparison to reference databases derived from other modern and ancient individuals. However, this is only possible if these references are available. Through studies of modern species, the number of these is increasing all the time, but they do set limits for the possibilities of aDNA studies.

Proteins – background and methods

Proteins have several functions in the living organism. Some of these are as structural proteins, that are the building elements of the cells and extra cellular matrix, such as collagen and osteocalcin. These are the main components of mammalian bone (and skin), while keratins are the main components of hair. As enzyms they act as catalysts for chemical reactions and as hormons they regulate processes in the body.

Proteins are linear polymers of amino acids (monomers) of which 20 different exist.

Short strands of less than 50 amino acids are called oligopeptides, whereas longer ones are called polypeptides or proteins. All amino acids are composed of a central carbon atom to which four chemical groups are attached: a hydrogen atom, a carboxyl group ($–COO^-$), an amino group ($–NM_3^+$), and the R group, which is different for each amino acid (Fig. 11).

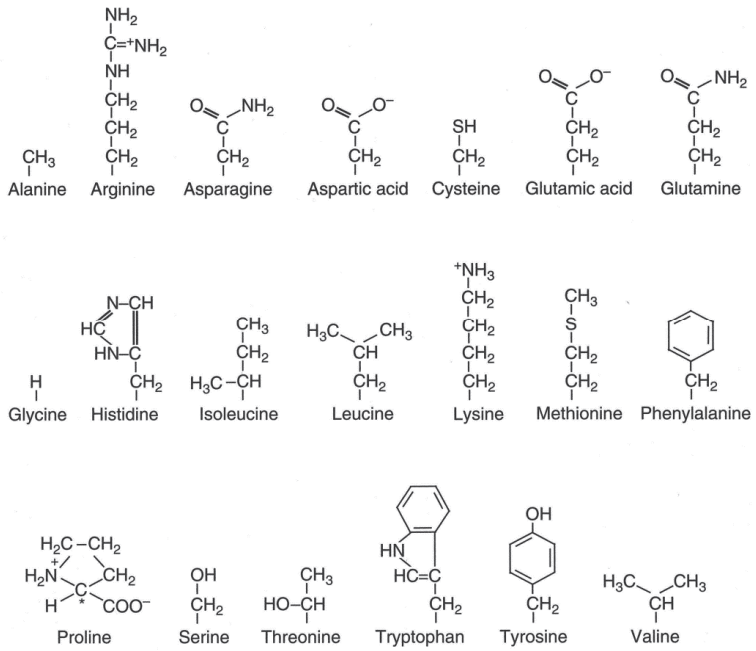

Figure 11. The R groups of the 20 different amino acids. For proline, the complete amino acid structure is shown, with the central C atom displayed as an asterix. After Brown and Brown 2011.

Proteins can therefore display a range of chemical and biological properties depending on the amino acids they are constructed of and the order of them (Brown and Brown 2011: 40-41). The amino acids are coded for by DNA and the synthesis of them is controlled by RNA. Proteins have four levels of structure of which the primary is the sequence of amino acids. The secondary, tertiary and quaternary determine the way amino acid strands are folded into 3D structures.

Tissues as bone and skin have been the predominant materials for paleoproteomics. Proteins can be extracted and digested to peptides (shorter strands of amino acids). The peptides can be analysed by a mass spectrometer (see later). The data from this will allow the identification of the peptides and proteins.

Typically collagens are the most predominant extracted proteins we will discover, as they are the most predominant biomolecules in ancient skin and bones.

The sequence differences of homolog peptides in different species can allow us to species identify materials by comparison to known sequences. Proteins are however not able to give informations on more specific levels such as breed or individual levels.

Preservation of ancient proteins

Proteins have been demonstrated to be superior to DNA with regards to longterm survival. The packing of collagen molecules was demonstrated to protect them from degradation processes (Collins et al. 2000, San Antonio et al. 2011). The elevated abundance of thermostable amino acid residues in type I collagens also contribute to its survival (Wang et al. 2012). Moreover, degraded samples from which DNA could not be amplified has been shown to yield ancient proteins (Schmidt et al. 2011, Brandt et al. 2014).

Contamination

Ancient proteins also face the risk of being contaminated. Contamination has however not been adressed as intensively in proteomics as in the field of ancient DNA. Contamination from modern contaminants in the laboratory can adressed in several ways. In our study, contamination of latex proteins from latex gloves was avoided by using only nitrile gloves for all preparation and analytical procedures.

All possible environmental contaminants recovered, such as actin, tubulin, keratins and keratin-associated proteins, as well as proteins commonly used in mass spectrometry facilities as standards or calibrants, as well as proteins that are highly conserved between species, such as histones, and all human proteins were excluded from further investigation. In the analysis, MaxQuant search against the entire NCBI protein database was moreover performed to exclude contaminants and evaluate random matches.

Cross contamination with other samples was adressed by using filtertips and in the analysis stage each nanoLC-ESI high resolution MS/MS run of an ancient sample was followed by a blank run to avoid carryover between samples.

At the time the samples were processed, no samples of *Artiodactyla* or *Perissodactyla* had previously been extracted for proteomics investigation and trypsin digested in the same facilities.

Mass Spectrometry-based proteomics

A mass spectrometer consists of three parts: an ion source, a mass analyser and an ion detector, though the nature of these can vary (Aebersold and Mann 2003, Fig. 12).

The samples reach the ion source in solid or liquid phase. In this first part of the mass spectrometer the analytes are ionized and vaporized to gas phase, before they are transferred to the mass analyser.

LC-ESI (liquid chromatography-electrospray ionization) is a method to ionize peptides as opposed to MALDI (Matrix-Assisted Laser Desorption Ionization), which ionized from solid phase.

The charge that the molecules receive allows the mass spectrometer to accelerate, control, and filter the ions throughout the mass analyzer to the detector. The ions encounter electrical and/or magnetic fields from mass analyzers, which alter the paths of individual ions based on their mass and charge (m/z). Commonly used mass analyzers include ToF (Time of Flight) which will be mentioned in chapter 4, quadrapoles, ion traps, and the electrostatic ion trap (Orbitrap) which is used in chapter 2.

Ions retained after filtering by the mass analyzers then hit the ion detector, that registers the number of ions at each m/z value (Aebersold and Mann 2003).

Tandem mass spectrometry (MS/MS) offers further analysis of specific peptides which are selected based on their m/z from the first round of MS. These are again fragmented and separated based on their individual m/z ratios in a second round of MS.

In chapter 2, LC-ESI and high-resolution tandem mass spectrometry (MS/MS) are used to sequence peptides instead of identifing them based on their masses. The latter approach, conventionally refered to as Peptide Mass Fingerprinting (PMF) (Henzel et al. 2003), is mentioned in chapter 4. PMF is a method to discriminate species by the difference in mass of a particular peptide, which is caused by the substitution of an amino acid between species. Such substitutions changes the mass of a peptide and allows the species identification of unknown peptides by matching their masses with peptide masses in a database such as NCBI. PMF (often referred to as ZooMS – Zooarchaeology by Mass Spectrometry) has been applied for species identification of proteins as keratin (see for instance Hollemeyer et al. 2002, 2007, 2008, 2012, Solazzo et al. 2013), osteocalcin (see for instance Ostrom et al. 2000), and collagen (Buckley et al. 2009, 2010, Kirby et al. 2013) from archaeological material.

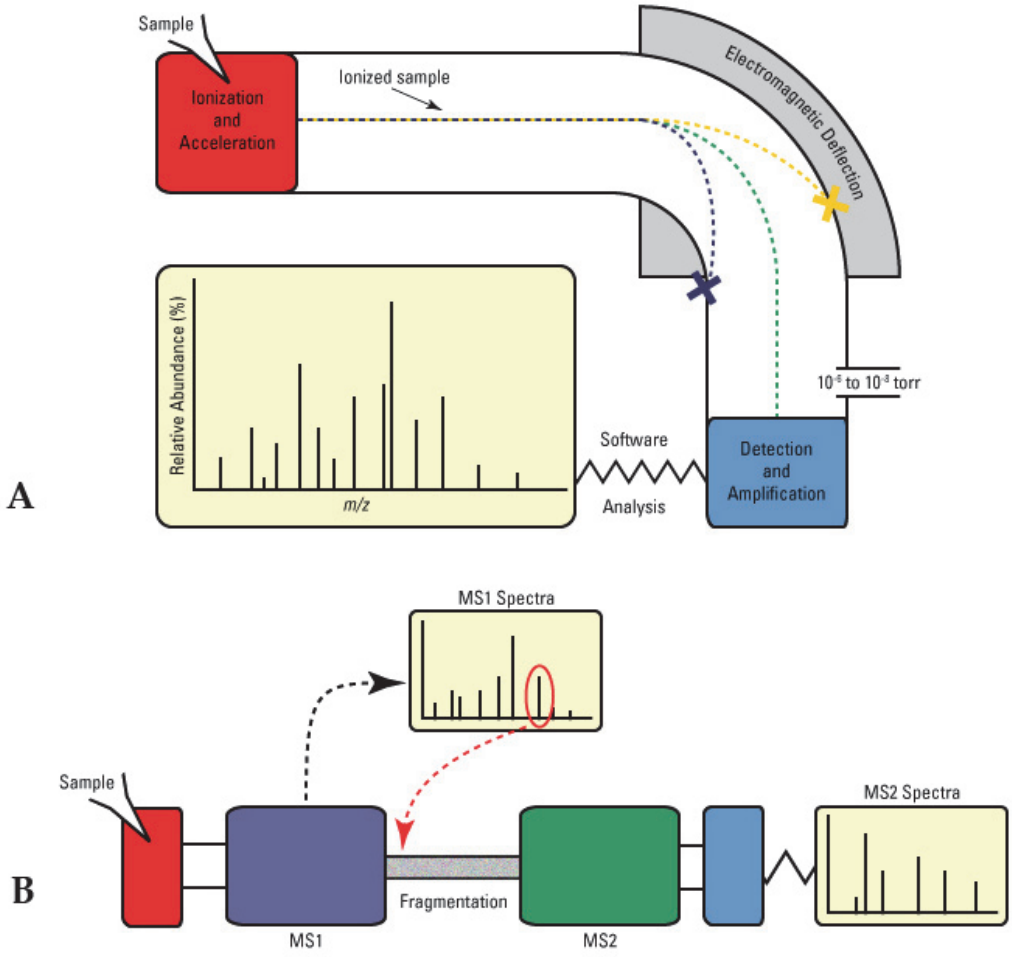

Figure 12. The principle of A. a mass spectrometer and B. tandem mass spectromety.

The advantage of PMF is that only the masses of the peptides have to be known, which is less time-consuming and expensive than sequencing. PMF is therefore ideal for large-scale applications. However, by sequencing proteins or peptides, also the amino acid composition is recovered. The maximisation of molecular recovery and data interpretation is crucial when applying invasive analyses to material of high cultural heritage value. Despite the necessity to sacrifice small parts of archaeological objects in the process, the collection, and public sharing, of the richest possible set of molecular information, consistent with technology and knowledge available at the time of analysis, is of infinite value for the understanding of our distant past.

PART II

Interdisciplinary studies of species identification and sheep wool development

Introduction

The production of a textile is a complicated process. It requires resources, including raw materials and labour, technology consisting of tools, know-how, and techniques to turn raw materials into finished products, and it is influenced by society as demonstrated by Andersson Strand et al. 2010 (Fig. 13). The focus of this chapter is wool as a raw material for textile production and its impact on textile production as well as on technologies and sociey.

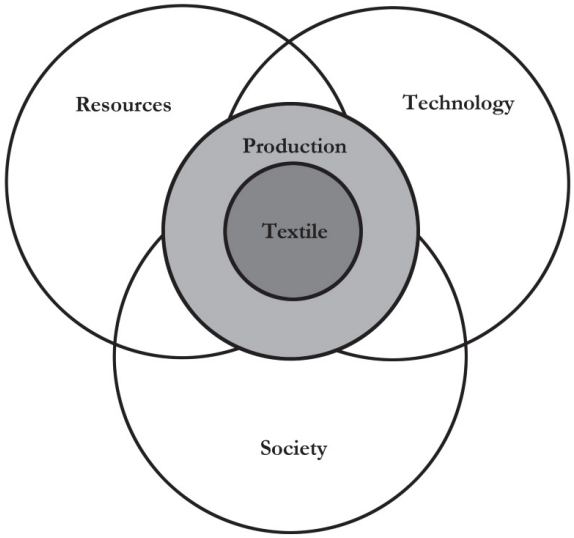

Figure 13. Model of textile and textile production as interaction between resources, technology, and society.

The onset and development of wool textile production is at the moment a popular topic in archaeology and is studied by several European research groups: TOPOI,[6] Creativity in the Bronze Age (CinBA),[7] ZBSA[8], and First Textiles.[9] As the onset of wool textile production had a massive socio-economic impact, it is not surprising that it is intensively studied. Wool became a desired raw material and the

[6] (http://www.topoi.org/group/a-4/). Several scholars in the TOPOI work on "The textile revolution".
[7] http://cinba.net/. As demonstrated in (Rast-Eicher and Bender Jørgensen 2013), this HERA-funded research project also investigates the oldest wool textiles of Europe.
[8] Zentrum für Baltische und Skandinavische Archäologie, http://www.zbsa.eu/news/news-2012/dfg-bewilligt-forschungsprojekt-zur-entdeckung-der-wolle
[9] (http://ctr.hum.ku.dk/economy/first_textiles/) This reasearch project within the Centre for Textile Research investigates the early textile production in Europe based on the preserved tool material.

production of wool textiles became an enormous economic factor (Waetzoldt 1972, Killen 2007, Michel and Nosch 2010).

The onset of wool textile production depended on the development of the raw material of which the textiles was made: the wool fibre. The development of sheep wool and the first production of wool textiles has been investigated from several sources including sheep bones, written sources, iconography and textile tools. This chapter investigates sheep and wool fibre development by combining archaeology, state-of-the-art textile research, and new genetic evidence with focus on prehistoric Danish sheep. Such an analysis not only characterises the raw material but also brings us closer to understanding sheep and wool as actors in selective breeding, processing of fibres, textile quality and technology and the use and impact of textiles in prehistoric Denmark.

First evidence from archaeology and textile research will be analysed, then the new genetic research will be presented.

Classification of the wild sheep species of the world

The genus *Ovis* comprises a large number of wild species and subspecies that differ by morphological traits such as body size, horn morphology, colour and pattern of the coat, chromosome diploid number, and geographical distribution (Rezaei et al. 2010: 315). Thus the number of possible ancestral species for domestic sheep (*Ovis aries*) and species believed to have contributed to its gene pool is large.

The taxonomy of wild sheep is controversial, and scholars have proposed several classifications during the last two centuries (summarized in Hiendleder et al. 2002). The International Union for the Conservation of Nature and Natural Resources adopted Valdez´ classification (Valdez: 1982, Shackleton and Lovari 1997: 13-14), a classification that distinguishes five species. However, the classification of Nadler et al. (1973), based on the numbers of chromosomes in *Ovis*, is also in use, as it distinguishes the greatest number of taxonomic entities (seven). Their classification is adopted here, to avoid confusion as many of the studies cited refer to this classification.

Nadler et al. (1973) identify the following seven species, here listed with chromosome diploid numbers: *Ovis nivicola*, the Siberian snow sheep, 2n=52 (still unstudied cytologically in 1973, Nadler et al. 1973: 117); *Ovis orientalis*, the Asiatic mouflon, 2n=54; *Ovis musimon,* the European mouflon, 2n=54; *Ovis canadensis*, the American Bighorn sheep, 2n=54; *Ovis dalli*, the Thinhorn or Dall sheep, 2n=54; *Ovis ammon*, the Argali sheep, 2n=56, and *Ovis vignei*, the Urial, 2n=58.

Evolutionary history of the genus *Ovis*

Analysis of the cytochrome *b* (Cyt *b*) gene in all wild *Ovis* species (except *Ovis nivicola*) has shown that the genus *Ovis* is monophyletic, i.e. a group formed of a species (common ancestor) and all its descendants. This genus has a Eurasian origin, in accordance with the fossil record (Rezaei et al. 2010: 324).

The American Bighorn (*O. canadensis*) and Thinhorn sheep (*O. dalli*) are monophyletic and form a monophyletic clade with the Siberian snow sheep (*O. nivicola*). This structure supports a migration of *Ovis* from Asia over the Bering Strait to North America, with later differentiation in Northeast Asia, Alaska/Canada and western North America (Rezaei et al. 2010: 324).

The analysis of Rezaei et al. (2010: 324) supports the existence of two Centralasian species, because *O. orientalis* and *O. vignei* both fall into monophyletic clades. On the basis of an analysis of the Cyt *b* gene, *O. musimon* clearly falls within the *O. orientalis* clade and must be considered both a subspecies and a wild remnant of the first domestic sheep that entered Europe (Rezaei et al. 2010: 324).

Given the calibrations used for the study, the separation of the Asian *Ovis* species from the Siberian snow sheep and the American *Ovis* species took place about 2,42 million years ago and the Asian species separated about 1,26 million years ago (Rezaei et al. 2010: 316, see Fig. 14). Therefore the geographical distribution of the wild *Ovis* species seen today has a long history and must have existed at the time of the domestication of *Ovis*.

The ancestor of domesticated sheep

The many existing wild species of *Ovis* has lead to many suggestions for the progenitor of domesticated sheep, *Ovis aries*, and the question has been confused even more by the different classifications of the species.

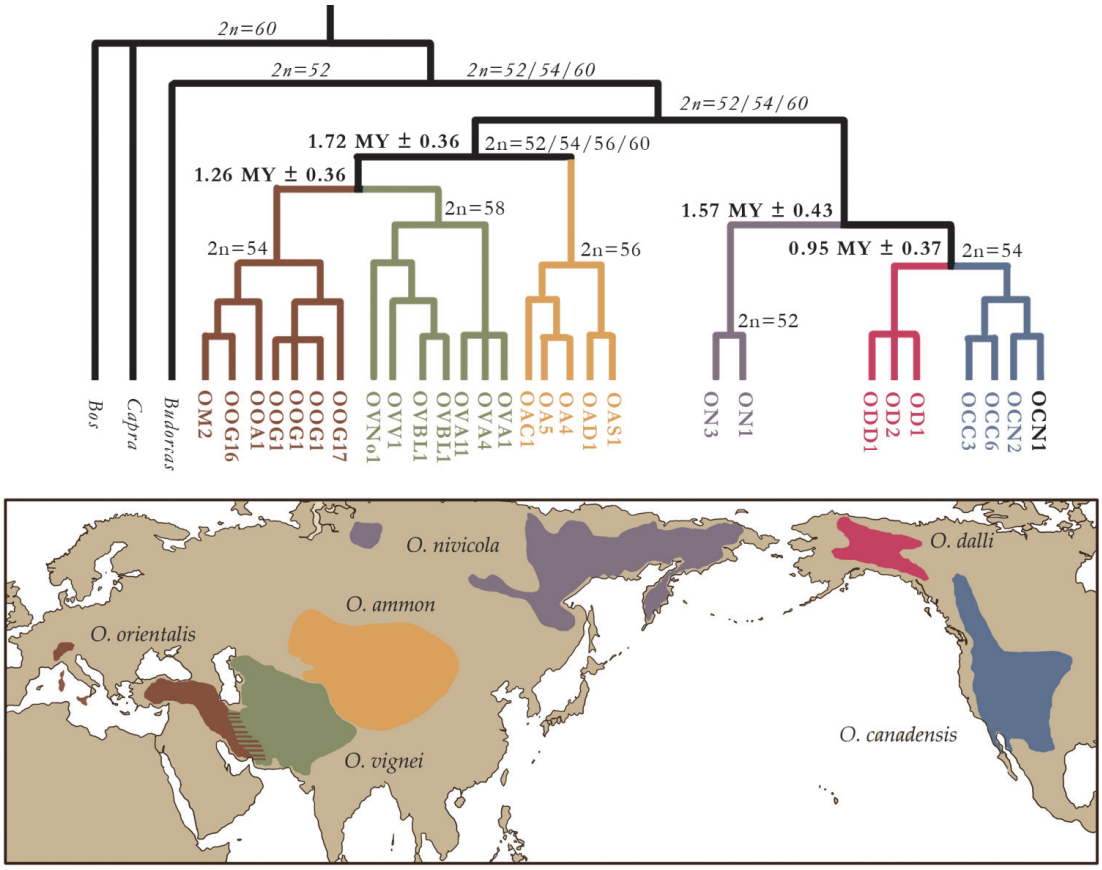

Figure 14. The evolutionary history and phylogeography of the six wild *Ovis* species according to the classification of Nadler et al. (1973). The seventh species in accordance to Nadler et al. (1973) *Ovis musimon* is reprecented by the blue areas in Europe. After Rezaei et al. 2010: 316. Graphics: Sidsel Frisch, modified after Rezaei et al. (2010: 316).

The attempts of identifying the progenitor of domesticated sheep are numerous and come from various disciplines. Historically, the evidence adduced has consisted in morphological traits and geographical distribution, later the number of chromosomes and molecular analysis have been included.

Charles A. Reed assumed that the domestication of sheep must have taken place in southwestern Asia as he states that *Ovis* is not present in Africa after the Pleistocene and in post-Pleistocene Europe. Based on this assumption Reed stated that domestic sheep derived from *Ovis orientalis* (defined as including both *O. orientalis* and *O. vignei*) which inhabit this area (Reed 1960: 135). Zeuner (Zeuner 1963: 135f) believed that *O. vignei*, the Urial, was the first to be domesticated. He stated that the Argali, *O. ammon,* later contributed to the gene pool of the domesticated Urial.

In 1971 Nadler et al. published studies of chromosome diploid numbers on wild sheep species (Nadler et al. 1971), that showed that the species had different chromosome haploid numbers (karyotypes). However, studies showed that species with different karyotypes can hybridicize in overlapping zones, resulting in chromosome diploid numbers of 2n = 54, 55, 56, 57 and 58 (Valdez et al 1978: 56, 58). This led Valdez to consider *Ovis orientalis* and *Ovis vignei* for one moufloniform species (*Ovis orientalis*) (Valdez et al. 1982).

As demonstrated, the studies of the chromosome numbers thus did not clarify sheep taxonomy and the ancestor for domestic sheep completely, and thus molecular phylogenies were needed. It has been generally accepted, that the ancestor should be found among *Ovis orientalis*, *Ovis ammon* or *Ovis vignei.* The largest problem with the genetic analysis is however, as we shall see, that the *Ovis orientalis* has often not been included in the material as it is not as well described as the other species (Meadows et al. 2011: 705). Several research groups have studied sheep phylogeny of which the more recent will be mentioned here.

Based on the analysis of mitochondrial DNA from 254 wild and domestic sheep, Hiendleder et al. 1998 stated, that *Ovis ammon* and *Ovis vignei* are too different from the domestic sheep to have contributed to its gene pool, and that its ancestor is not yet identified (Hiendleder et al. 1998: 117). This is a statement that the group retains, and their later analysis show that *Ovis ammon* and *Ovis vignei* fall as clades clearly separated from *Ovis aries* (Hiendleder et al. 2002: 901: figure 4). They now see *Ovis orientalis* as the most likely progenitor for domestic sheep, even though this species is still not included in the material. Meadows et al. 2007 included *Ovis orientalis* in their studies of sheep haplotypes along with *Ovis ammon* and *Ovis vignei.* However none of the domestic haplotypes cluster with any of the wild species and Meadows et al. thus see the question of the progenitor as unresolved (Meadows et al. 2007: 1378). In Bunch et al. 2006 *Ovis aries* og *Ovis musimon* fall within the clade of both *Ovis orientalis* and *Ovis vignei* (Bunch et al. 2006: 27-28), which implies that both of these species could be the ancestor of domesticated sheep. However, Rezaei et al. 2010 (including samples of hybrids between the species and subspecies of *O. orientalis* and *O. vignei* mentioned in Valdez et al. 1978 and the sequences of *O. orientalis* included in Bunch et al. 2006) show that *Ovis orientalis* and *Ovis vignei* fall into clearly distinct lineages and should thus be considered as separate species. The study moreover shows that *Ovis musimon*, the european mouflon, which, as we will return to, has turned out to be a neolithic domestic population that ran wild, falls under *Ovis orientalis.* This implies that *O. Orientalis* is the ancestor of domestic sheep. This result should be considered at the standpoint of the research and *O. orientalis* is at the present considered the most likely candidate to be the ancestor of domesticated sheep.

The number of domestication events of *Ovis*

Modern sheep breeds have been studied to investigate the number of domestication events of sheep (Hiendleder et al. 1998, Pedrosa et al. 2005, Meadows et al. 2007, Meadows et al. 2011). Meadows et al. 2007 found evidence for five ovine mitochondrial lineages: haplogroup A, B, C, D and E. The highest diversity of these is found in the Near East and decreases to Europe, which we shall is in accordance with the archaeological evidence of the origin and spread of domestic sheep

The dating of the divergence of the lineages, 0,26 million years is considerably older than the time of domestication pointed to by archaeological evidence around 8-9000 BC (Meadows et al. 2011: 705). It is therefore highly unlikely that the haplogroups demonstrate 0,26 million years of differentiation following domestication, but should rather be seen as molecular evidence for several domestication events of distinct sheep populations. Meadows et al. consider it most likely judging from the five haplogroups that at least five domestication events of sheep have taken place, probably from different wild populations, that however most likely all were of the *Ovis orientalis* species (Meadows et al 2011: 705). The place of these events has been proposed to be the Middle East and Central Asia.

Archaeological evidence for the place of domestication of *Ovis*

The Asiatic mouflon, *Ovis orientalis* (the most likely ancestor of domestic sheep) is adapted to life in the mountains. The fossil record lacks evidence for Pleistocene presence of *Ovis orientalis* outside the Middle East and Central Asia, e.g. in Europe (Clutton-Brock 1999: 70) This indicates that *Ovis orientalis* was originally restricted to the Middle East and Central Asia (Beneckes 1994: 229). One would therefore expect that the initial domestication of sheep took place in this area.

Before entering the discussion on where the oldest domesticated sheep derive from, it is necessary to first define what is understood by "domesticated", as this term has been intensively debated by researchers in the field.

Melinda A. Zeder, who has worked extensively with domestication of especially sheep and goat, states that there is general agreement that domestication involves a relationship between humans and the targeted population of animals or plants (Zeder 2006a: 105, Zeder 2012: 162). She views domestication as a process that begins with a wild population and ends with a population completely managed by humans and separated from the original wild population (Zeder 2006a: figure 1). It is the precise point within this process at which one defines the population as domesticated that causes debate. The nature and degree of human control that characterises the relationship between humans and animals has also been debated. Some definitions put emphasis on humans as the controlling part, whereas others view it as a mutualistic relationship that both parts benefit from (see discussion and references in Zeder 2006a,

Zeder 2012). The extent of morphological and genetic changes in the domesticated animals has also been viewed as defining domestication (Zeder, 2006a: 105). Because morphological and genetic changes occur with a delay from the first domestication, the opposite point of defining domestication from a change in human behavior has also been argued (Zeder 2006a: 106).

In this thesis, domestication is not viewed as an event that clearly separates wild animals from domesticated ones, but as a process in which the relationship between animals and humans evolved. The full extent of this relationship is characterised by ongoing interaction between animals and humans, including the adaptation by humans and their society to the new resource. Through such a definition, evidence of dometication must therefore be attested in both animals and human society.

Traditionally, domestication has been identified by changes in animal biogeography and biodiversity seen in prehistoric bone assemblages, evaluation of osteomorphology and osteometry, as well as changes in demographic profiles of animals. Lately, new methods as isotopic and genetic analysis have been applied. In the following, the most frequently applied ways of identifying domestication and their possible pitfalls will be examined.

Biogeography and biodiversity

The occurrence of a species outside its natural habitat and an increase in frequency of a previously less important species has been seen as an indicator of domestication (Zeder 2006a: 109). Peters et al. for instance document the replacement of the Persian gazelle by ovicaprids at four settlements in Southeast Anatolia sites during PPN (Pre Pottery Neolithic, 10000-7000 BC). They see this as an indication that hunting had been replaced by animal husbandry (Peters et al. 2013: 84-91). The data presented by Peters et al. 2013 seem very convincing, Zeder (2006a: 109-110), however, argues, that the intensification of frequency of a species in bone assemblages may simply reflect changes in hunting strategy and not domestication. She also states that the geographic range of prehistoric animals is poorly known.

Osteomorhpology and osteometry

Evaluation of morphopological traits on animal bones and bone meassurements have been used extensively as documentation for distinguishing wild and domesticated animals.

A decrease in size has often been singled out as the main indicator of animal domestication. This is because size reduction has been interpreted as a response to human management and its controlled breeding, diet quality and reproductive isolation, or because smaller sized animals were easier to manage and were therefore selected. However, the size of wild animals can also change naturally as a response

to climate and the environment and it also differs according to the animals sex and age. Failing to account for such factors can lead to erroneous conclusions of domestication (Zeder 2006a: 109). Zeder e.g. argues that the size reduction seen in some materials is caused mainly by a different distribution of male and female individuals towards a higher ratio of females. Because of sexual dimorphism such a shift could appear as a general size reduction if the sex distribution is not recorded (Zeder 2011: 226). It thus seems that size reduction is a factor that can not alone be used to identify domestication, but that several markers are needed to build a better case. This is for instance the approach of Arbuckle and Aticis study (2013) that seeks to combine biometric data with demographic profiles.

Although morphological traits are definitely useful in investigations of domestication, they will only begin to occur after a certain period of time and therefore do not enable us to identify the very first domestication. They are thus only able to provide a *terminus ante quem* (Peters et al. 2013).

Investigation of domestication from demographic profiles

As mentioned above (concerning Arbuckle and Atici 2013), evaluation of demographic profiles in bone assemblages are often used as indicators of domestication. As will be demonstrated in the following, they are a powerful tool indeed. Due to general problems working with bone assemblages including taphonomic factors, representativity (see theory and method section), and methodological problems, one must, however, be careful when using demographic profiles. Before introducing the possibilities of using such profiles, their background and possible pitfalls will therefore be introduced.

Demographic models of animal exploitation

Payne (1973) presents models for the *kill-off pattern* in a sheep or goat population, that is the sex and age at death of the slaughtered individuals, which he states would reflect the purpose of domesticated prehistoric sheep or goats. The theory is that it would be economically advantageous to slaughter animals at different ages and of different sex according to the desired product. Payne presents a model of how the kill-off-pattern would look in flocks kept for respectively meat, milk and wool (Payne 1973: 282-284, see Fig. 15).

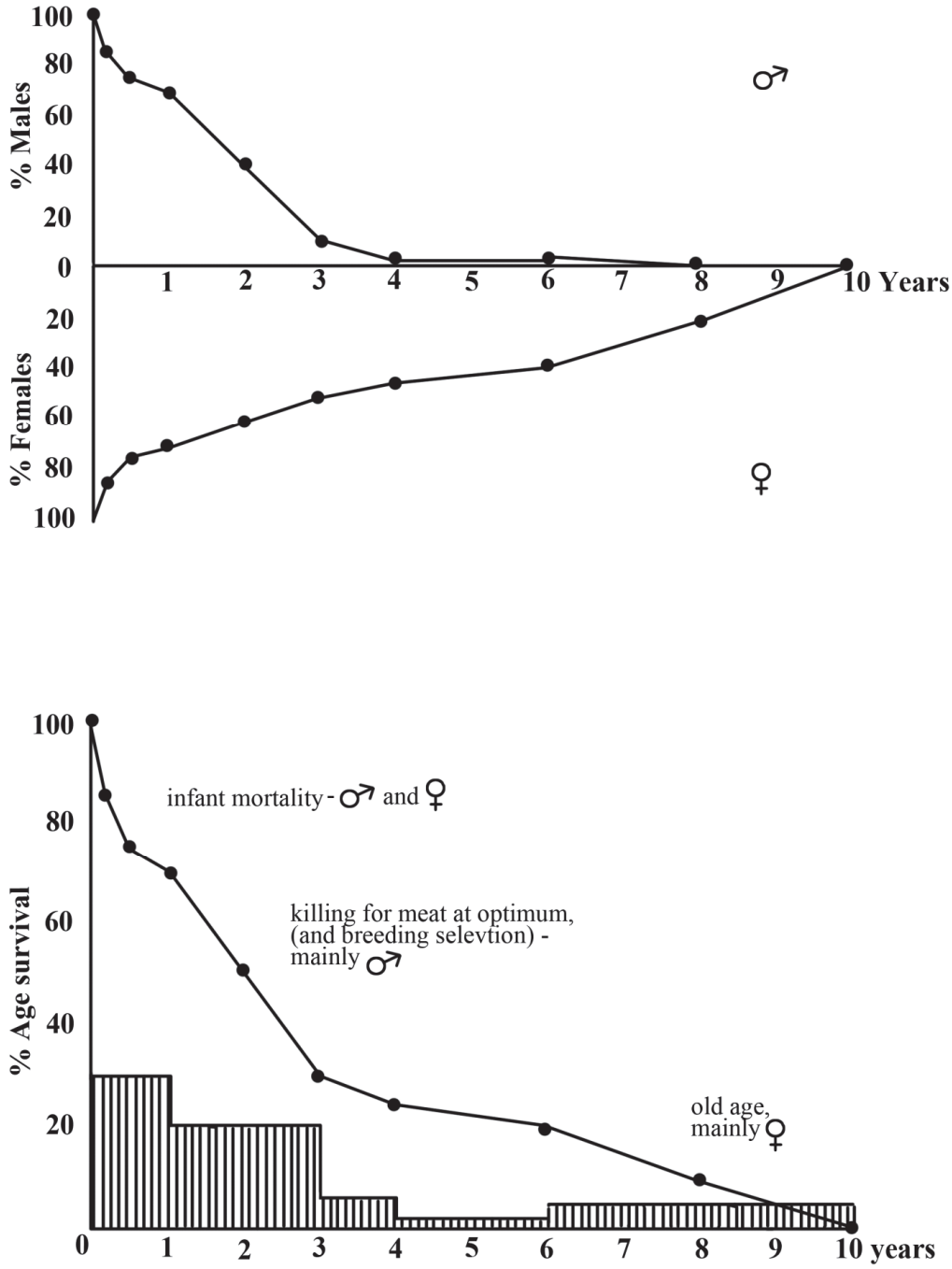

Figure 15a. Kill-off pattern. Models of sex and age of slaughtered animals in herds kept for meat. After Payne 1973.

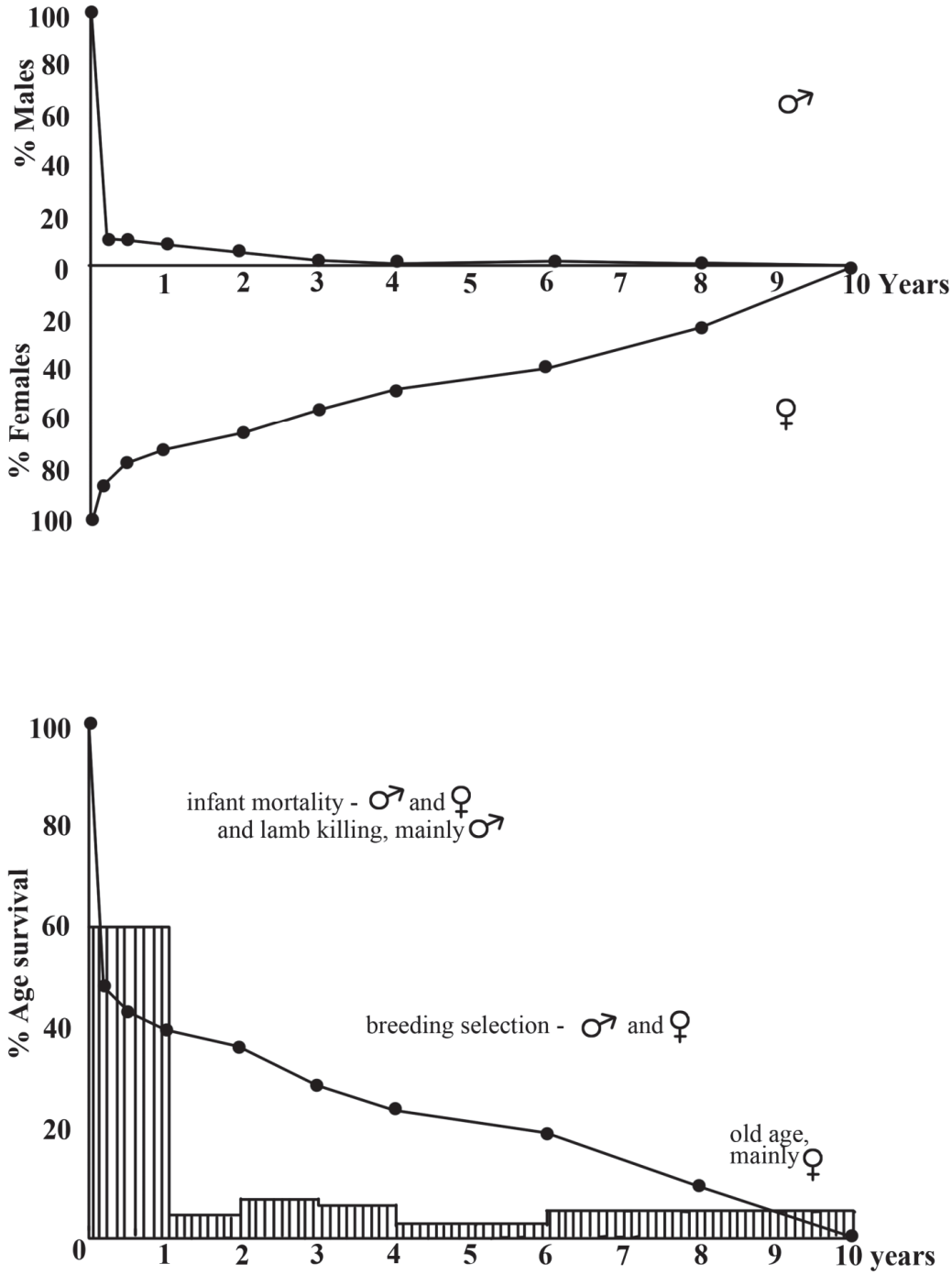

Figure 15b. Kill-off pattern. Models of sex and age of slaughtered animals in herds kept for milk. After Payne 1973.

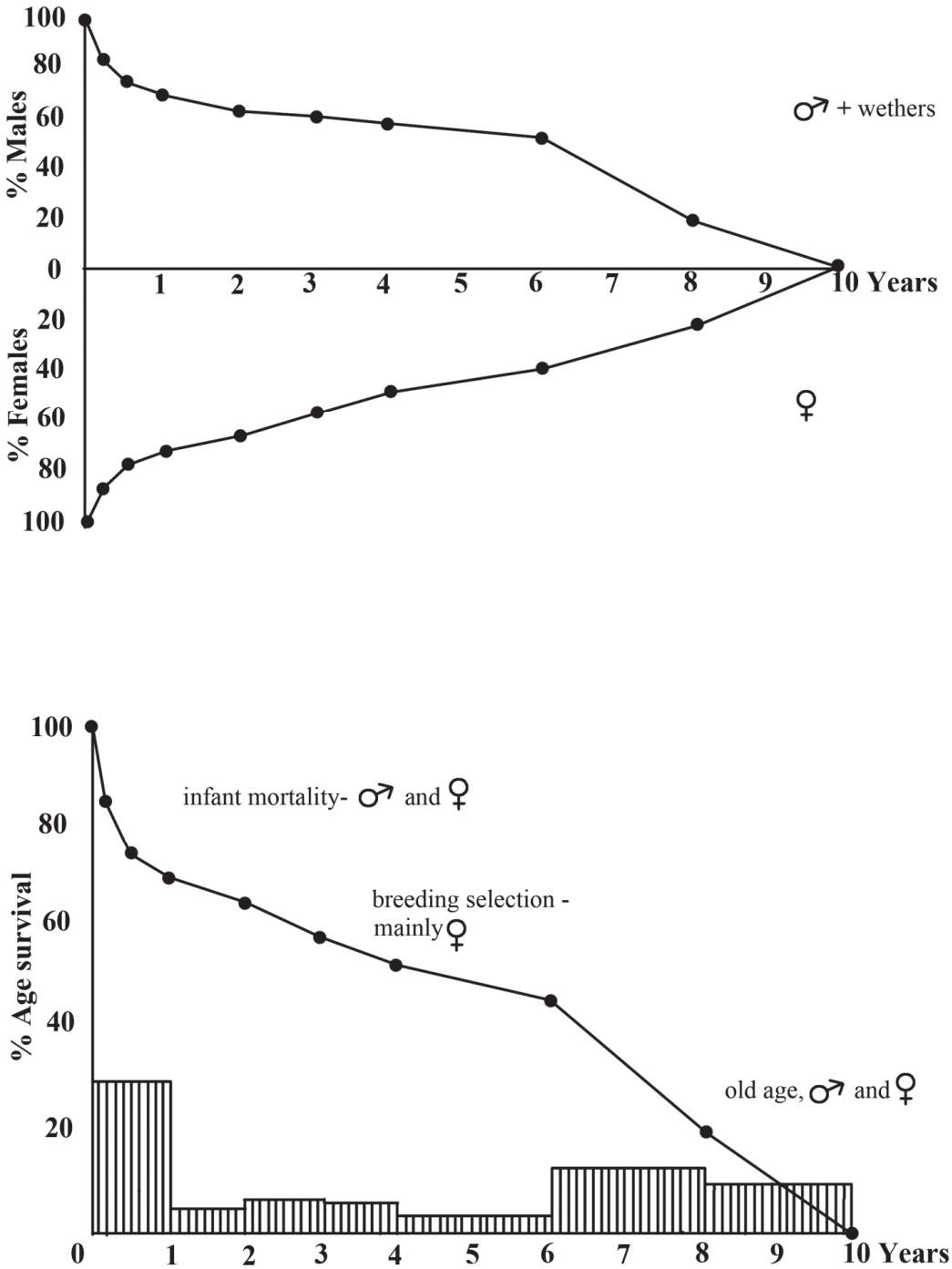

Figure 15c. Kill-off pattern. Models of sex and age of slaughtered animals in herds kept for wool. After Payne 1973.

If meat production is the aim, most individuals are killed when they reach the weight-gain optimum (1-3 years of age) and only a smaller proportion is kept for breeding. If milk production is the aim, the surplus of lambs not needed in the production is killed as soon as milk production is not endangered. Especially young male individuals are slaughtered. For wool production the population is expected to reach an older age. Males not needed for breeding would be castrated (Payne 1973: 281). Based on Paynes model one should thus expect many rams, as they give the best wool, but also many wethers, and generally older animals on a site specialized in wool production (Wigh, 2001: 91).
Kill-off-patterns have often been used on sheep populations to argue that the population was kept primarily for meat, milk or wool production. The considerations of using kill-off patterns to identify prehistoric animal exploitation is first that they build on modern economic ideas of animal husbandry and optimisation. Second, prehistoric sites would rarely display populations exploited for one product only. For instance, wool producing sheep must most probably have been slaughtered for meat as well and it is likely that two or several products were equally important.

Apart from theoretical considerations regarding the application of kill-off-patterns, there are also methodological considerations regarding the material on which the kill-off-patterns are based. Establishing kill-off patterns requires thorough zooarchaeological investigations of bone materials including at least species identification, a count of the Number of Identified SPecimens (NISP) or Minimum Number of Individuals (MNI) (Lyman 2008),[10] and an evaluation of the sex and age of the individuals (Wigh, 2001: 44-52). Sex and age estimations are methodologically problematic, as they are based on modern references which may not correspond to prehistoric populations. Moreover, not all bones can be used for sex and age estimations and, a more problematic issue, there is no standard for which bones to use, which makes it difficult to compare materials. Therefore the refinement and correlation of demographic profiles is at the centre of scholarly attention (Zeder 2006b: 174, Arbuckle and Atici 2013).

Sex and age profiles have long been considered as the best evidence for domestication. Profiles demonstrating slaughtering of mainly young animals corresponding to the meat pattern has been seen as an indication of herded populations. A bone assemblage deriving from a selected hunting strategy targeting young individuals would however be hard to distinguish from a one deriving from herded

[10] Whether to use MNI or NISP has been debated greatly. Both methods have advantages and problems. The use should depend of the purpose of the study. In this case I prefer MNI, in order to be able to test different individuals and construct kill-off patterns.

populations with focus on the slaughter of young male individuals, or if such an expected herding pattern even existed (Zeder 2006b: 174).

Though one should in my opinion be careful with testing prehistoric sheep bone assemblages against modern economic models for sheep herding, there may be general economic considerations that applies to sheep herding and makes demographic profiles valuable. However, I believe that they cannot stand alone, as there could be other explations for them as demonstrated above. I therefore believe that they should be supplemented with more information such as morphological changes and especially the archaeological context, as will be described below.

Investigation of domestication with genetic methods

Genetic methods have also been applied in order to distinguish wild and domesticated populations genetically and it has been attempted to identify genetic markers which can be used to trace the domestication process (Zeder et al. 2006).

Mitochondrial DNA has been studied extensively in this respect. Particularly its non-coding parts which are not subject to selection but retains its mutations can be used to investigate the relationship between populations. MtDNA also has the advantages that it evolves 5-10 times faster than nuclear DNA which is useful when dealing when short periods of time as for instance 10.000 years (Zeder et al. 2006: 142).

On the downside, mtDNA fails to document admixture between populations as it does not recombine (Larson and Burger 2013: 200) and therefore there are several questions which cannot be answered by studying mtDNA. Despite its lacking resolution, mtDNA remains a valuable tool as there is a rich worldwide dataset available for comparison and computing models have been developed for it in order to test hypotheses of population genetic history.

There is no doubt that improvements in next generation sequencing techniques to study nuclear DNA from archaeological samples will yield great new insights in ancient population dynamics and domestication as it provides the opportunity to study population diversity, structure and admixture (Larson and Burger 2013: 201). As with morphological changes, genetic changes also occur with a delay and can thus not be used to document the initial domestication.

Investigation of domestication from cultural context

The above described methods are all theoretically well-founded and experimentally confirmed approaches to identifying animal domestication. Nevertheless, they also have pitfalls which makes it hard to identify domestication based on any single method. Therefore, I believe that the safest way to

argue for animal domestication is by the combination of two or more of the described methods. Moreover, in order to trace the impact of domesticated animals on humans, the cultural context of suggested first domesticated populations should be included in the analysis.

Several scientific methods have recently been taken into use in order to supplement traditional archaeological methods. These include isotopic analysis to identify changes in foddering and herd mobility (Pearson et al. 2007, Meiggs 2009) and geochemical identification of penning and use of animal dung (Arbuckle and Atici 2013: 219). Also archaeological evidence as changes in site architecture, establishment of enclosures, changes in tools and technology can support a change in subsistence economy. A combination of traditional zooarchaeological analysis with investigations of the archaeological context is for instance demonstrated in Olsens investigation of horse domestication (Olsen 2006). Such large-scale investigations are, as Scarre states, easier to conduct on long-lived sites at which one can compare between phases:

"The best evidence comes from long-lived settlement sites, where we can watch for changes of size, changes in morphology, changes in the age and sex ratios, and changes in the spectrum of animals providing meat. It is usually the case that bones from the domesticated herds dominate samples, where they are present, and the range of animals hunted is much reduced." (Scarre 2009: 228).

Documenting animal domestication

Traditional and newer methods for documenting animal domestication have been discussed above. Based on different theories on how domestication may be recognised, they all provide tools for the identification of such a transition. The interpretation of data through these methods are, however, not unambiguous. Several of the phenomenons can be explained by natural processes. Therefore, the combination of several methods would provide the strongest evidence for animal domestication.

Also following the above stated definition for domestication applied in this thesis, the counter adaptation of humans is equally important to define domestication, which means that the inclusion of the archaeological context and its changes following animal domestication are highly important.

Above, the author stated that domestication should be viewed as a process. Through the above account it becomes clear that the very first domestication will be hard to document. This is perhaps nevertheless not that important, as it is the gradual adaptation and counter adaptation which is of interest. And this is what we shall look for in the next chapter that aims at finding evidence for early sheep domestication.

The earliest domesticated sheep

The presence of sheep bones in bone assemblages dating to the Paleolithic demonstrate, that sheep were already a resource in the Middle East before their domestication (Zeder 1999). Sheep were

therefore most likely domesticated to have a stable meat and fur supply. Wool can be ruled out as the purpose for the domestication, as *Ovis orientalis* did not have the characteristic woolly fleece of modern sheep, but were hairy with only a very thin underwool (Ryder 1983, see Fig. 16).

Figure 16. The coat of *Ovis orientalis*. Copyright Aykut Ince.

At present, the earliest evidence for a transition to sheep domestication comes from two sites, Zavi Chemi Shanidar and Hallen Çemi, in the north western Zagros (Fig. 17). Around 12.000-11.500BP sheep bone assemblages at the two sites change at this time to a focus on 2-3-year-old males. This has been argued to demonstrate a transitional hunting stategy maintaining the presence of females and young animals and keeping a flow in the supply of young males (Zeder 2011: 227). This strategy qualifies as a transition to domestication, between game management and herd management, as it does not quite represent control of a herd, but however increases the availability of animals for slaughter. Arbuckle and Atici point to that an "initial diversity" characterized early ovicaprid management of the 9th and 8th millennia BC, which makes them suggest further scrutinisation of bone assemblages from 10th and 9th millennia sites as Hallen Çemi, Körtik Tepe, Zawi Chemi Shanidar, Qarassa and Nachcharini for initial local management strategies (Arbuckle and Atici 2013: 232, see Fig. 17). At Nevali Çori the transtition to herding has been argued to be complete by 10500 BP where both changes in demographic profiling and animal size has been detected (Peters et al. 1999). Also Cafer Höyük (10.300-9.500 BP), Aşikli Höyük (10.200-9.500 BP), and Abu Hureyra (ca. 9.600 BP) have been pointed out as settlements with early sheep domestication based on changes in demographic profiles og sheep bone assemblages (Zeder 2011: 227). During the Early Epipaleolithic (18.000-12.000 BC), Southwest Asia was occupied by hunter-gatherers. The settlements vary from small sites of seasonal to the first sedentary sites (Scarre 2005: 205-206).

Also the settlements of the Late Epipaleolithic display great variation from small camp sites to large permanent settlements (Scarre 2005: 208). Zawi Chemi Shanidar and Hallen Çemi were earlier argued to display a transition to sheep management. These sites both display stone-built houses demonstrating sedentarization and tools for processing seeds (Scarre 2005: 211-212). A wish to keep a stable supply of sheep would be most beneficial for a sedentary population as seen for Zawi Chemi Shanidar and Hallen Çemi. Moreover, the economy at these sites also display an adaptation to crops. The archaeological evidence at these sites therefore support the hypothesis that a transitional strategy between sheep hunting and herding was performed here.

Over the Pre-Pottery Neolithic A and B (9600-8800 BC and 8800-6900 BC) settlements grew and cereals that appear cultivated morphologically become more frequent. The previously mentioned settlement Abu Hureyra develops from a small Epipalaeolithic settlement to a large Pre-Pottery Neolithic village with mud-brick houses. Also the increasing wear on human molars and increasing dental caries support a larger reliance on cereals (Scarre 2005: 212-231). These changes demonstrate a larger reliance on domestic resources and fit well with the changes in the demographic profiles of

sheep. However, in Abu Hureyra the transition from foraging to farming was a process that lasted several centuries.

After 7500 BC the wide-spread practice of young male kill-off across south-western Asia suggests deliberate management strategies. The emergence of phenotypically domestic ovicaprids in the mid-8th-millenium BC along with severe changes in the animal economies in general in the Near East demonstrates that domesticated sheep are now present in the Near East (Arbuckle and Atici 2013: 232). Also the archaeological record of the Ceramic Neolithic (6900-6000 BC) demonstrates severe changes in settlement type, architecture, and in cultural life.

In the sixth and fifth millennia BC sheep bones show further morphological changes as hornlessness in ewes and shortening of the limb bones, indicating domestication (Clutton-Brock 1999: 75). In South-West Asia, early villages with farming economies grew and became more complex after 6000 BC. They spread into southern Mesopotamia, where they led to the rise of the first cities (Scarre 2005).

Sheep domestication seems to be a process that stretched over thousands of years and varied between settlements. It started out as a transitional strategy from hunting to farming and ended in forming part of an indispensable base for emerging cities.

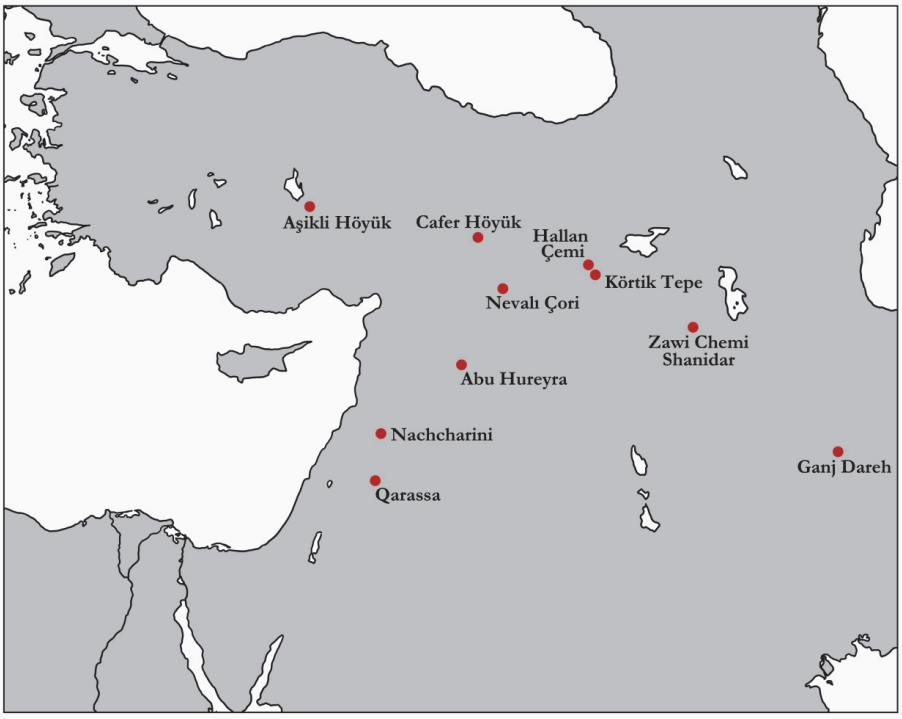

Figure 17. Sites in question of the first sheep domestication. Graphics: Sidsel Frisch.

The development of sheep wool

As with the initial domestication, there are several difficulties connected to talking about "the first wool". First, wild sheep already have wool, even though it is just a thin underwool covered by kemps. The question is rather then one of the first spinnable wool. Kemp are too short to be spun. Hairs can be spun, but give a harsh and bristle thread with stiff ends sticking out. Wool on the other hand is ideal for spinning (Barber 1991: 21). The coat of wild sheep therefore needed development in order to be useful for spinning.

Changes in prehistoric sheep could therefore provide information on when and perhaps how sheep changed, but not necessarily human adaptation to this. The occurance of a new phenotypic trait and the selection may moreover have taken many generations.

Depending on the focus, the development of sheep wool and human adaptations to this is not something that can be dated precisely, but is, as domestication, something that should be viewed as a cultural historical process that displays different degrees of development.

There are several sources of evidence that can yield information of the first sheep wool. As will be described in the following paragraphs, this can be divided into indirect evidence, including studies of sheep bone assemblages, written sources, pictorial evidence and sculptures, and direct evidence comprising textiles and sheep skins.

Indirect evidence for the development of sheep wool

The earliest evidence that has been suggested to indicate sheep wool is a clay statuette from the neolithic settlement Tepe Sarab in Iran dated to around 5000 BC (Fig. 18). The statuette depicts a sheep with V-shaped regular lines on the side, which have been interpreted as evidence for a wool bearing sheep (Ryder 1987: 104). The sheep bones from Tepe Sarab demonstrate that majority of the sheep were kept to an adult or mature age, which could support the use of sheep for secondary products as early as 5000 BC in Iran (Bökönyi 1977: 25).

Figure 18. The sheep figurine from Tepe Sarab, Iran, with V-shaped incisions. After Benecke 1994: 137.

From the ancient city of Uruk, located in present-day Iraq, comes thousands of clay tablets with writing. The oldest of these are dated to 3100 BC (Nissen 1986: 317). 48 of these tablets were analysed by M. W. Green and deal with animal husbandry. For the first time, sheep are distinguished and described in signs as "wool-sheep", proving that sheep with fleece were present in Uruk in the late 4th millenium BC (Green 1980: 4, see Fig. 19).

In Europe, Linear B tablets from late Bronze Age (Mycenaean) palaces in the Greek mainland, textile workshops, textile workers, and the number of sheep in specific flocks and their expected yield of wool are listed. This is evidence of a well-developed wool industry controlled from the palaces. In 14th and 13th century BC Greece (Killen 2007).

Figure 19. Clay tablet from Uruk, showing the mark for wool-sheep. After Green 1980: 20.

Direct evidence of sheep wool

Textiles dating to around 6000 BC found at one of the worlds earliest cities in Central Anatolia, the Neolithic Çatal Höyük (Hodder 2006), have been claimed to be made of wool (Helbaek 1963). This statement has later been rejected for several reasons. The straight and parallel fibres and the clean breaks of fibres are characteristic of flax but not wool which is more wavy and less parallel and has a

cellular structure which would yield less clean breaks. The lack of medium and coarse fibres which would appear in early wool, and the lack of disulphide reaction during the chemical tests which would appear for wool but not for plant fibres due to its chemical composition also indicated that the textiles are flax. (Ryder 1965).

The so far earliest known sheep wool is from the Bronze Age settlement Shahr-i Shōkhta, Eastern Iran (Good 1999: 110) and a Bronze Age burial mound from Novosvobodnaya, North Caucasus (Shishlina et al. 2003) dating to respectively 3200-2800 BC and 3700-3200 BC.
A sheepskin dated to to the 4th millenium BC comes from the predynastic site el-Omari in Lower Egypt (Greiss 1955: 228-229). Michael Ryder's studies of the fibres showed that the wool had a continuous range of fibres from 12 to 110, which is comparable with hairy rather than kempy sheep (Ryder 1983: 109-10). This skin demonstrates that the sheep fleece had developed from the wild kempy fleece to a hairy fleece in Egypt.

Prehistoric sheep and wool in Denmark

There are several sources to prehistoric sheep and sheep wool in Denmark. These include sheep bones dating from around 4000 BC and textiles of sheep wool which occur much later from the Early Bronze Age period I (1700-1500 BC).
Textile tools provide evidence of textile technologies and the archaeological context in general provides evidence for textile production and the importance of textiles. Finally also natural scientific methods as strontium isotopes from sheep wool and genetic analysis contribute to the investigation of prehistoric sheep and sheep wool in Denmark. The following paragraphs will focus on evidence of sheep and sheep wool from the Late Mesolithic to the Late Iron Age with parallels to contemporary Europe.

Sheep bones from Danish prehistory

The earliest sheep bones from Denmark are C^{14} dated to the transition between the Mesolithic and the Neolithic around 4000 BC. New C^{14} dates point to that sheep were already present in the Late Mesolithic Ertebølle Culture (Sørensen 2014). Early Neolithic finds of sheep bones are, however, very few and only single finds.
In the Middle Neolithic Period, bones of ovicaprids (sheep and goat) generally make up 10-20% of the bone assemblages (Nyegaard 1985), which demonstrates that sheep become a more important resource during the Neolithic. Artefacts such as jars for cheese production moreover demonstrate that secondary products like milk start being utilised in the Neolithic.

The frequency of ovicaprid bones in bone assemblages increases to between about 20-50 % in the Bronze Age of which the majority of the species identified bones are made up by sheep (Nyegaard 1998).

In his dissertation from 1996 (second edition 1998) on faunal remains from Danish Bronze Age, Georg Nyegaard states (based on the material available in 1996), that age profiles of Bronze Age ovicaprids do not suggest that the main purpose of ovinoculture was the utilisation of wool, but that this was a secondary product to meat (Nyegaard 1998: 152). However, the horn core of an adult late castrated ram (wether) indicates that wool from local sheep was utilised, even though wool quality would have been improved by early castration (Nyegaard 1998: 45). Castration is often seen in connection with wool production as wethers produce the best wool since ewe wool quality declines during pregnancy and lactation (Wigh 2001: 91).

In the Bronze Age, the frequency of species represented in bone assemblages varies between Danish regions (Nyegaard, 1998: 155). These regional differences continue in the Pre Roman Iron Age. In Northern Jutland sheep and goat make up 59% of the species identified bones, whereas they only make up 19% on the islands (Kveiborg 2008: 66).

Kveiborg argues that the heath of Northern Jutland was well suited for sheep grazing and that this might be the reason for the high frequency of sheep in this region, though other factors such as human preferences may also have played a role (Kveiborg 2008: 82-83).

Sheep are frequent through the entire Iron Age. Apart from their obvious use as food as demonstrated by for instance the food offerings in graves from the Roman Iron Age (Lund Hansen 1995, Hatting 2000, Gotfredsen in prep.) their purpose for primary or secondary products is evaluated based on kill-off-patterns.

Based on the frequency, kill-off-patterns and size of Danish sheep, Nyegaard for instance argues that wool production was not common in Denmark until the Pre Roman Iron Age (Nyegaard 1998).

Based on zooarchaeological investigations of the animal bones from the early strata of the city of Ribe, dated to the 8th Century AD, Tove Hatting argues that the sheep from Ribe are of a particular breed (Hatting 1991: 48-50). She states that the horn cores of the sheep from Ribe are very different from roughly contemporary horn cores of sheep found in Denmark. Moreover, allmost all sheep from Ribe reach maturity. This age profile is different from what is seen at Viking Period Haithabu and the Medieval towns Svendborg and Viborg where sheep were slaughtered at a young age. Taking the finds of textiles, spindle whorls, and loom weights (Bender Jørgensen 1991) into account, Hatting argues that the Ribe sheep were herded for wool and that they may represent a particular breed (Hatting 1991: 49-

50). It thus seems that at least from the late Iron Age we may expect that specialised breeds for particular products were found in Denmark.

Early Danish textile technology

Textiles of plant fibres were produced in Denmark from at least the Late Mesolithic, which is demonstrated by plant fibre textile fragments from Tybrind Vig dated to 4300 BC (Bender Jørgensen 2013). Stone Age textiles are rare in Denmark (Bender Jørgensen 1992: 159-160), but the few finds indicate, that a well-developed textile technology based on plant fibres existed (Mannering and Skals 2013). So far no finds of textiles from the Danish Stone Age demonstrate woven fibres. The finds display techniques of twisted and plaited cords, which enters into sewing and plaiting techniques.

Loom weights begin to appear in the archaeological record in the Late Neolithic period for instance in a pit at Nørre Holsted in Southern Jutland (Rindel 1993).
These loom weights demonstrate that weaving technology was known and performed in the Late Neolithic period, which is before textile finds can demonstrate a woven textile technology. This indicates that the production of woven textiles was introduced earlier than hitherto assumed.
It is uncertain if animal or plant fibre was utilised for weaving in the Late Neolithic. The donut-shaped loom weights are quite heavy (500-600g). Loom weights can provide evidence for the type of thread it was used for (Andersson Strand 2010). Heavier threads require heavier loom weights (pers comm. Eva Andersson Strand). Thus a woollen cord with many fibres or a tightly spun woollen cord would have been well suited for this type of loom weights.

Society and cultural connections in the Danish Late Neolithic

In the Danish Late Neolithic I (2400-2000 BC) metal objects such as gold sheet ornaments, gold lunulae, and copper axes begin to occur in the archaeological record. These new objects have their closest parallels in Western Europe (Vandkilde 2007: 24-29) and have been found particularly frequently in Northern Jutland. Here Beaker pottery further demonstrates contacts with the western European Beaker groups (Vandkilde 2007).
Imitations of for instance bronze daggers in flint from the Late Neolithic and Early Bronze Age demonstrate that European artefact types were know and desired and thus copied in locally available material (Lomborg 1973: 18-19).
Also dress fasteners, amber buttons, and bone dress pins, are new in the archaeological record. Amber buttons have their primary distribution in northern Jutland and have the closest resemblance to types

found in Western Europe (Ebbesen 1995: 235-239). The pins on the other hand are found mainly in Eastern Denmark and have their closest parallels in Central and Eastern Europe (Ebbesen 1995: 223-230).

Ebbesen argues that the first occurrances of pins and amber buttons indicate a change in costume to one produced of sheep wool (Ebbesen 1995: 249-250). However, he fails to take into account that garments of plant fibres could also be fastened by dress pins. Plant fibre textiles were, as previously mentioned, already known at this time. He emphasizes that the time of the introduction of these new dress fasteners coinside with the introduction of weaving indicated by the first finds of loom weights. From the late Neolithic dress pins are found in all preceding time periods.

The artefact types found in Jutland and the islands differ, and it seems that Jutland is mainly influenced by the western European Beaker groups whereas the islands are mainly influenced by Eastern and Central Europe (Vandkilde 2007)

In LN II (2000-1700 BC), the metal import increases and the majority of this now comes from the eastern European Únětice culture, that covers eastern Germany, Poland and the Czech Republic (Vandkilde 1996: 298).

At the Hungarian tell site Százhalombatta-Földvár, the importance of sheep increased after 2000 BC. The majority of these reached an adult age, and about a third of them were
males, which indicates the wool production became more important at the site after 2000 BC (Vretemark 2010).

Finds of wool textiles from the Czech Republic dating to Bz A (2000-1500 BC) demonstrate that the Únětice culture at this time produced textiles of wool (Belanová-Stolcová 2012: 309). Apart from the changes in dressing inspired from Europe demonstrated by new dress fasteners, the textiles themselves may have been desired exotica. Wool textiles could have been present in the countries that were of main influence to Denmark in this time period, indicating that the first wool or wool textiles arrived in Denmark around this time.

Sheep wool textiles from Denmark

It is generally assumed that the first Scandinavian sheep had the same coat as wild sheep (Ryder 1983, 1988), although no clear evidence for this has yet been presented. However, based on this asumption, sheep breeds with wool must either have been imported or the wool developed locally over time.

Though sheep are present in Denmark from the transition between the Mesolithic to the Neolithic, it is not until the first period of the Early Bronze Age period I (1700-1500 BC) that textiles of wool occur. Bender Jørgensen lists a single find from period I, Briksbøl (Bender Jørgensen 1986). In period II this number increases to 75 finds, also including textiles from oak coffins. Many of these graves have been dated with high precision by dendrochronology (Christensen 2006). An early oak coffin with textiles is Sønder Ønlev 8 at the beginning of period II, dated to sometime after ca. 1468 BC. More well known finds of textiles from period II include the three graves from Guldhøj (ca. 1389 BC), Egtved (1370 BC), and Borum Eshøj A and B (respectively ca. 1348 and ca. 1344 BC) (Randsborg and Christiansen 2006: 115-117). Others have been dated archaeologically by their artefacts. Such graves include Skrydstrup, which most likely dates to the Early Bronze Age period III (1300-1100 BC) (Randsborg and Christiansen 2006: 117). Bender Jørgensen counts 41 finds of textiles from period III after which the number decreases to 10 from the entire Late Bronze Age (Bender Jørgensen 1986: 189-192). This most likely mirrors the development in burial costums where cremations take over.

Textiles of wool predating the Early Bronze Age are not attested. The lack of wool textile finds could be explained by conditions of preservation in grave types dating to the preceeding time periods: burials in stone cists and reburials in megalith tombs of the Late Neolithic and in mounds that precede the amazing oak log coffin graves of the Early Bronze Age period II-III. Also the lack of metal objects in the majority of the Neolithic period could play a role, as it is often in association with these that textiles are preserved in later periods.

Studies of wool fibre development

Studies of how sheep wool changes over time are based on studies of wool fibres. Wool quality characteristics include among others fibre diameter, crimp (number of bends per unit length), yield, colour, and staple strength and length (Rast-Eicher and Bender Jørgensen 2013: 1224). Of these traits, fibre diameter is the single most important charateristic determining wool quality and the quality of textiles produced from it in both prehistory, history and the present. Investigations of changes in wool quality over time have therefore been performed by measuring the diameter of wool fibres from archaeological textiles.

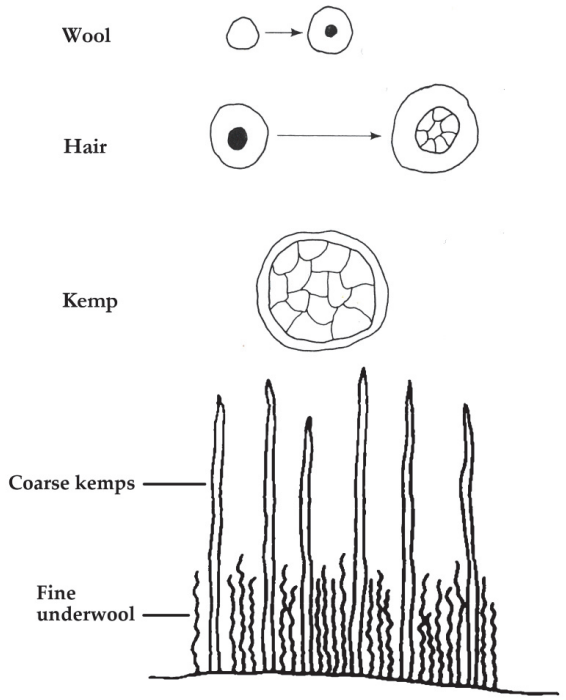

Figure 20. Sheep fleece with kemps and underwool and the difference between kemp, hair and wool.

Ryder and Stephenson distinguish three types of fibres in the sheep coat: kemp, hair, and wool, that differ in structure and size (Fig. 20, Ryder and Stephenson 1968: 282-286). Wool fibres are crimpy and rarely have medullas (central cores), while kemps or guard hairs are coarse with broad medullas, and hairs are the intermediate between these with narrow or interrupted medulla. Wool fibre diameter is measured in microns (μ),equivalent to one-thousandth of a millimetre. This means that the lower the number of microns, the finer the fibre. Kemps range from about 100-250μ and hairs range from 50-100μ. Wool is classified as medium between 30/35-60μ and as fine under 30μ (Barber 1991: 21).

Michael Ryder's extensive work on wool fibres has established an important base for studies of the development of wool fibres in archaeology. Ryder studied wool development by measuring the wool fibre diameter in sheep fleeces of modern breeds and wool from archaeological textiles. Ryder used measurements of 100 fibres from a sample to produce histograms displaying the distribution of fibre diameters. Based on the histograms, Ryder proposed a hypothetical evolutionary scheme for sheep wool development. He distinguished six "typical fleeces" based on fibre measurements of sheep fleeces of modern breeds (Fig. 21).

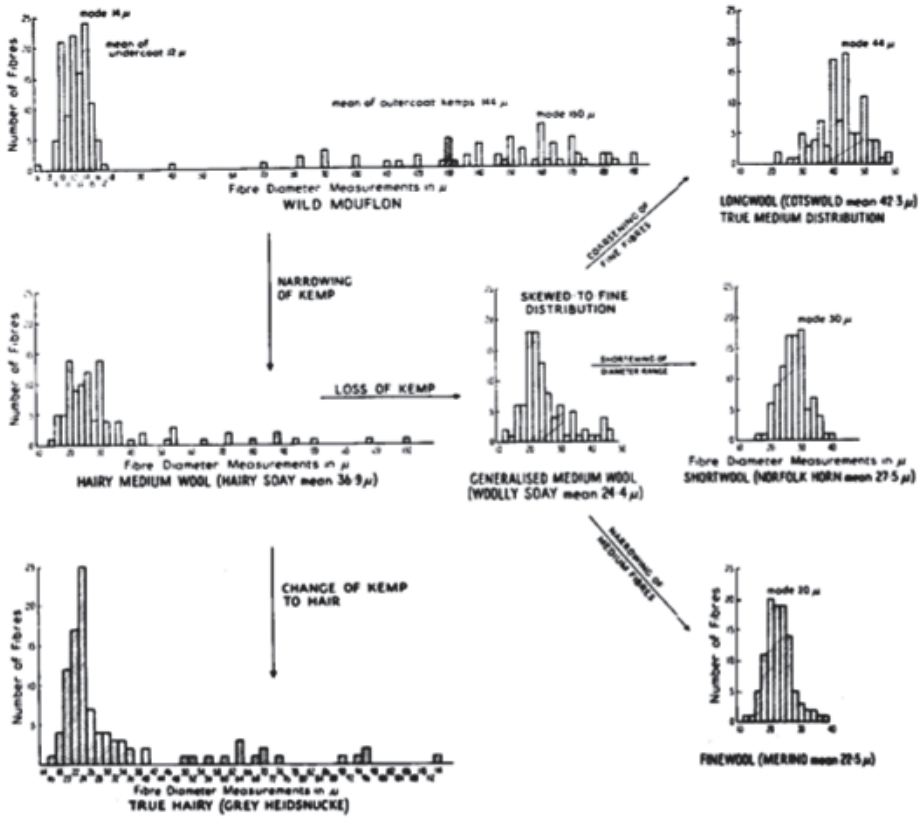

Figure 21. Ryder's suggestion for the evolution of wool. After Ryder 1983, fig. 3.36.

The most primitive fleece was represented by the wild mouflons, which he argued had a coat that resembled the coat of Neolithic sheep. The next evolutionary step was represented by the fleeces of the hairy and the woolly Soay breed. These breeds are believed to have developed very little since prehistory and are therefore likely to represent what early wool breeds would have looked like. These are the fleece types Ryder refers to as *"hairy medium wool"* and *"generalised medium wool"*. Ryder suspected, that the coat of the mouflon, containing coarse kemps and fine underwool, went through a narrowing of the kemps that turned them into the less-coarse fibres seen in the coat of the hairy Soay sheep. Ryder further suggested that the loss of the hairy fibres turned them into wool of medium diameter (*"generalised medium wool"*). Ryder suggests that the fine, long and short wool seen in modern breeds as Merino, evolved from this medium wool by a loss of the medium sized fibres and a narrowing of the fibre diameter.

Although Ryder's studies have been of great importance for the research of wool, they have been critisised by several scholars over the past years (Good 1999 139-140, Christiansen 2004, Gleba 2012, Rast-Eicher 2008, Rast-Eicher and Jørgensen 2013). Christiansen explicitly states: *"For the model to be valid, the fibre composition of both yarn and fleece must accurately reflect each other"*, meaning that the model would only be valid if no sorting processes of the wool took place. Ryders does state that fine wool was a result of separating the coarse fibres from the fine either by the plucking of wool or sorting by hand (Ryder 1988). Ryder does not, however, fully acknowledge a sorting process, which precludes the connection between yarn and fleece types. Textile researchers have moved to viewing the wool of textiles as representing a *chaîne operatoire* of processes it has passed through (Andersson Strand 2012). These include the plucking or shearing of wool, maybe restricted to specific breeds, animals, and even bodyparts of sheep, the sorting of wool into qualities e.g. after colour, staple length etc., combing and perhaps even more processes that we do not know (Andersson Strand 2012, Mannering and Gleba 2012).

Therefore, the idea of recognising Ryder's fleece types in a textile is today refused, as archaeological textiles are believed to display a sorted and processed wool and not the composition of fibres in the sheep fleece it derives from (Rast-Eicher 2008: 126). However, the uninterupted range of fibre diameters in histograms can nevertheless provide clues of the original fleece and be used for comparison between wools (Gleba 2012, Skals in press).

Changes in Danish sheep wool from the Bronze to the Iron Age

Ryder has investigated the fibre diameters of a number of Danish Bronze Age wool samples (1969, 1983, 1988). He recognises two types of fibre compositions: hairy medium and fine fibre diameter distribution (Ryder 1988: 139). Hairy medium is recognised by a combination of fine fibres and hairs in textiles from Lille Dragshøj, Rønhøj, and Trindhøj, whereas only fine fibres were recorded in the analysed textiles from Trindhøj, Guldhøj, Borum Eshøj, Sandbaek, and Skrydstrup (Ryder 1988).

The uninterupted range of the fine fibres from both categories of textiles are however very similar (Ryder 1988: 139). Taking the critique of Ryder's catagorisations into account, the differences between the mentioned textiles are most likely caused by the sorting processes of the wool and not evidence of different wool types.

Irene Skals has measured the fibre diameter in Danish wool textiles from the Early Bronze Age, Pre Roman Iron Age and Roman Iron Age (Skals in prep.). Here the width of the uninterupted fibre range varies with only a few microns between Bronze Age and Iron Age with no clear pattern. Moreover

outliers are found in both Bronze Age and Iron Age textiles. Judging from Skals´ data there is no obvious change in Danish sheep wool from Early Bronze Age to Iron Age and wool quality is a matter of sorting rather than a change in sheep fleece types. According to new analysis more developed wool types do not occur in Denmark until in the Roman Iron Age (1-400AD) (Mannering in prep.a).

Comparative material from Europe

Compared to the Danish textiles, fibre measurements of textiles from Italy and the Austrian Alps demonstrate that sheep wool in this region went through major changes from the Bronze Age to the Early Iron Age (Gleba 2012). The range and mean diameter of the fibres demonstrate a development from a very fine underwool and very coarse kemps to a coarser and more uniform fleece with no kemps. Whereas all kemps could in theory be removed during sorting, a change in the diameter of the uninterupted range of fibres is a good indication of different sheep fleece types.

Moreover, Gleba suggests the presence of different sheep breeds from the first millenium BC, as distribution curves of fibre diameters from this period vary in range (Gleba 2012: 3653).

Bender Jørgensen and Rast-Eicher investigated wool samples from three European regions: Hallstatt, Scandinavia, and the Balkans (Rast-Eicher and Bender Jørgensen 2013). Both sheepskin and textiles have been preserved in the Austrian Bronze Age and Iron Age salt mines of Hallstatt. The sheepskins of the Bronze Age display short, coarse fibres and fine underwool. The Bronze Age textiles display a range of fibres similar to the skins but with fewer coarse fibres and kemps. This makes the overall quality finer and demonstrates that sheep wool was sorted before textile production. Some of the Bronze Age wools do, however, appear different from the wool seen in the sheepskins, indicating that they were imported from elsewhere. Compared to Scandinavian wool, the Hallstatt textiles and skins display a coarser wool, leading Bender Jørgensen and Rast-Eicher to propose that these wools derive from sheep with different types of fleece than the Scandinavian.

The Iron Age sheepskins and textiles from Hallstatt were more variable in quality and colour than the Bronze Age wools. For the textiles this suggests that sorting processes were refined. Also in the Iron Age some of the wools seen in the textiles from Hallstatt do not correspond to the wools seen in the sheepskins. Also in this time period import of raw material or textiles thus seems likely (Rast-Eicher and Bender Jørgensen 2013).

New strontium isotope analysis further complicates the question of fleece development, as they show that prehistoric textiles could contain wool from different geographical regions (Frei et al. 2009b: 1969-1970). Based on these results textiles could contain wool from not only sheep from different regions

but also sheep of different breeds. This finding is of great importance for our interpretations of fibre diameter histograms. Wool from several breeds could bias our interpretations if these are based on the assumption that only one breed is represented. If use of wool from several breeds in one yarn is proved to be common we should rethink our interpretations of the histograms, though one may also speculate that only similar wool types would have been combined. Nevertheless it makes little sense to attempt to match fibres from textiles with a fleece type (Rast-Eicher 2008: 124). Wool from textiles should therefore rather be viewed as representing a combination of a wool quality and a sorting process chosen for the specific need.

Developments of wool textile technology in Denmark in the Bronze and Iron Ages

During the Bronze and Iron Ages, sheep and wool textiles became more abundant in Denmark. The wool fibre does, however, not seem to change before the Roman Iron Age.

Though the fibres did not change, textile production underwent several changes. Textiles dating to the Pre Roman Iron Age display several new elements compared to the Bronze Age. Both tabby and twill weaves, stripes and check patterns are present and analyses of dyestuffs have shown that many of the textiles were dyed in red, blue and yellow colours (Mannering 2009, Mannering et al. 2012, Berghe et al. 2009, 2010).

In the Roman Iron Age, diamond twill and tablet weaving was introduced and the number of threads per centimeter gradually increases. Also new tailored garments previously only known from the areas south of Denmark are introduced and the use of dress accessories such as fibulae become more widespread (Mannering et al 2012). These new trends are perceived to be the result of influences or inspiration from the costume traditions of Central Europe (Wild and Bender Jørgensen 1988).

Another change is that shears begin to appear towards the end of the Pre Roman Iron Age. Shears may have been used for multiple functions but cutting wool is argued to be one of the main ones (Henriksen 2009).

Primitive sheep breeds have a fleece that moults. This means that the wool was removed from the animal by plucking. Ryder argues that the cutting of wool initiated a development of the wool towards a non-shedding (1987: 117). More developed breeds do not moult and therefore their fleece has to be cut by shears or knives (Mannering et al 2012: 6). This means that less wool is lost during collection and that the time of collection can be shortened. The appearance of shears could thus indicate changes in the sheep and their wool.

Denmark and Europe 500 BC – AD 400

While several sheep breeds seem to exist in Europe during the first millenium BC, this is not demonstrated by fibres from Danish archaeological textiles dating to the Pre Roman Iron Age (see above).

In the Pre Roman Iron Age, Denmark is less influenced from continental Europe than in the preceding Bronze Age and following Roman Iron Age. Part of this is caused by the introduction of iron. This raw material was naturally present in Denmark, and copper and tin from the continent were therefore not needed (Kaul 2009). The disconnection with Central and Southern Europe is visible in the archaeological record as only few continental European objects make their way to Denmark (Kaul 2009).

At the end of the Pre Roman Iron Age, political conditions changed in Europe due to the expanding Roman Empire and Germanic tribes penetrating the southern borders, as well as an increase in Danish contacts with continental Europe (Kaul 2009). These contacts are demonstrated by many imported objects from the Roman Empire (see for instance Lund Hansen 1987, 1995).

Also the agriculture experienced changes due to influences from the Roman Empire including the introduction of hens and new cereals (Aaris-Sørensen 1988: 222, Helbæk 1970).

Pliny the Elder describes sheep from different regions of Italy with wool of differing fineness and colour. Also Columella distinguishes sheep breeds from different regions and comments on their wool (Gleba 2008: 74). These sources supports the existence of different breeds with different fleece qualities in the beginning of the Common Era in Italy (Gleba 2012: 3643).

Fibre histograms in textiles from Bronze and Iron Age Hallstatt (Rast-Eicher and Bender Jørgensen 2013) and Italy from the first millenium BC (Gleba 2012) support the statement that different wool types were present in Italy and Austria.

Specialisation of wool production is not indicated in Denmark at this point in time, but seems to appear later. Specialisation is of particular interest for this study, I hypothesize that a specialised production would require a large amount of raw material of certain qualities, as described in Greek and Mesopotamian sources. Signs of a specialised production could therefore indicate that also specialised breeds were present.

According to Danish zooarchaeological evidence different sheep breeds were also present in Denmark from at least the Late Iron Age (Hatting 1991, see earlier in this chapter).

Specialisation of wool production

To be able to discuss a specialised production, it is necessary first to clarify what is understood by specialised production. Specialisation in textile production has been extensively addressed by Eva Andersson Strand (Andersson Strand 2003, 2007, in prep.), who distinguishes four different degrees of specialisation in the Scandinavian Viking Age: household production, household industry, attached specialist production, and workshop production for trade (Fig. 22). The first category merely covers the needs of the household whereas the second produces a surplus of textiles for trade though this is not a full time occupation. The two last categories are more specialised forms of production, employing specialists and producing products of higher quality of which the last does so to the greatest extent.

Household production • Production solely covers the household's own needs • Household members possess knowledge and skills needed • Raw materials commenly accesible • Knowledge of manufacturing processes widespread	*Household industry* • Production scale beyond needs of producers • Organised at household level • Surplus used for trade, exchange or tax • Production when spare time is available • Work not full time occupation
Attached specialist producton • Production by specialists • Craftsmen supported by and dependent on patron • Work main occupation on full time basis • Specialist's skills enhanced by full-time occupation • Better quality products • High quality products as desirable gifts • Control of skills add to patron's power	*Workshop production for trade* • Direct production for market • Items practical and standardised • Production volume high • Great demand for products • Work full time occupation • Time costs per item reduced to minimum

Figure 22. Model of specialisation of textile production, after Andersson Strand 2003.

The model is based on the production in the Viking period and may not be suitable for time periods with completely different types of societies and organisations. However, Andersson Strand's model highlights important thoughts on the organisation of production and will be used here as a point of departure for a discussion of specialisation in Danish prehistory. Some of the parameters listed are hard to recognise archaeologically. However, the time spent on textile production and the amount of

products produced and their diversity are parameters that appear relevant to characterize production in all time periods. These can in some cases be investigated archaeologically. Andersson Strand and Mannering argue that in the Roman Iron Age the place of the weave, in either separate buildings or in the longhouses, could indicate whether textiles were produced full time or in the evenings (Andersson og Mannering 2010). Pit houses have been interpreted as workshops for weaving (Sørensen 1993). This has however been questioned by Gebauer Thomsen who argues that many pit houses do not fulfill the criteria for textile production, and that they could have had multiple functions such as dwellings (in the summertime) and workshops for other crafts (Thomsen 2010). It is therefore questionable if these structures can be related only to textile production as previously stated.

Andersson Strand sees the great diversity of the textile tools in Birka as an indication of a diverse production aiming at producing textiles for various purposes and for a larger market (Andersson 2003, 2007). Moreover, textiles and tools that exceed what would be needed for the household indicate that the production was aimed for the market. Although textile tool materials from Danish settlements are rarely well recorded, their presence and number in combination with finds of textiles and house structures with textile tools can be used to evaluate if a production of textiles exceeding household production could be argued. This would indicate a larger demand of wool and it is here that we perhaps should expect to find specialised wool breeds, if such existed.

The early city Ribe (see also earlier in this chapter) displays several such traits. Apart from the sheep bones which have been interpreted as displaying a specific wool breed and with a kill-off pattern differing from contemporary sites, Ribe has yielded a large amount of textiles and tools for textile production.

Archaeological conclusion

Sheep were introduced to Denmark shortly before 4000BC. At this time, technologies to produce textiles from plant fibres were already known. Weaving, however, seems to be introduced in the Late Neolithic when loom weights begin to appear. Changes in jewellery style and the introduction of dress pins also indicate the costume changes at this time. A natural conclusion is that it could coincide with the introduction of a new fibre for textile production: wool, as this was already utilised in the Únětice culture with which Scandinavia had close contacts. That wool has not been documented in the Late Neolithic could be connected to the burial costums of this time period, which are less likely to preserve organic materials than the oak coffins of the Early Bronze Age.

The attestation of loom weights indicates that a local production of woven textiles took place. Sheep, however, do not make up a large part of the zoological material and, although increasing in frequency

in the Bronze Age, it is not until the Pre Roman Iron Age that kill-off-patterns demonstrate a more targeted production of wool (Kveiborg 2008).
This raises the question if the textiles of the Early Bronze age were locally produced from local wool and if this was perhaps also the case for even earlier textiles. Scandinavian Bronze Age textiles are very distinct and uniform and recognisable by their plain weave, thick even yarn, and 3-5 threads per centimeter (Mannering in prep.b). This indicates that at least the textile production was local and so standardized that they were perhaps only produced a few places. Future strontium isotope analysis will however be useful in order to clarify if also the wool was local. However, strontium isotope analysis of a textile from Voldtofte and the Huldremose II garment dating to respectively the Late Bronze Age and the Pre Roman Iron Age demonstrates that plant fibres and wool or sheep were in fact imported (Frei et al. 2009b, Bergfjord et al. 2012).

In the Late Pre Roman Iron Age, shears come into use in Denmark, indicating that sheep wool is no longer of the primitive non-shedding type. This coincides with the changes in sheep wool fibre diameter in the Roman Iron Age indicated by new wool fibre analysis. This indicates that sheep wool changed around the Common Era.
This could be linked to the increasing contacts with Europe which already had different sheep types in the first millenium BC. At this time the costume and the use of accessories as well as agriculture changes, most probably due to impact from contacts with the South.

DNA analysis of prehistoric Danish sheep

As demonstrated above, much research has been carried out on prehistoric sheep and textiles. However, despite these excellent studies, several questions remain unresolved. The type of sheep that initially came to Denmark is still uncharacterised and the time and way Danish sheep diversified to include the many breeds know from historical periods is likewise uncertain. The time of the development from the primitive fleece of wild sheep to a quality that could be used for textile production is also unknown.
The focus of this study was to characterise the development of ancient Danish sheep and sheep wool using genetic methods based around ancient DNA analyses. The genetic results are in later paragraphs compared with state of the art knowledge from the fields of archaeology and textile research.
The author carried out the laboratory work and interpretations, whereas bioinformatics was performed by Jose Alfredo Samaniego and Luca Satriani Ermini.

Previous studies of ancient DNA from sheep and sheep wool

Studies of the control region and/or the cytochrome b gene of the mitogenome of modern domesticated sheep breeds from a wide geogrphical range have identified five haplogroups: A, B, C, D, and E (Wood and Phua 1996, Hiendleder et al. 1998, 2002, Guo et al. 2005, Pedrosa et al 2005, Meadows et al. 2005, 2007, 2011, see also earlier in this chapter). As the split of the haplogroups are dated much earlier than the initial sheep domestication (150-170.000 years ago for the split between haplotype A and B), the haplogroups are considered each to represent a domestication event (Pedrosa et al. 2005).

Haplogroup A and B are the most frequent and have been identified in sheep from all sampled geographical regions. Haplotype A is particularly frequent in Asian sheep whereas haplogroup B dominates in European sheep (Wood and Phua 1996, Hiendleder et al. 1998).

Haplogroup C is less frequent but have been located within both Asia, the Fertile Cresent and Europe (Guo et al. 2005, Pedrosa et al. 2005, Tapio et al. 2006, Pereira et al. 2006).

Haplogroup D and E are the least frequent and have only been identified in samples from Turkey and Caucasus (Tapio et al. 2006, Meadows et al. 2007).

Several studies have been carried out on archaeological sheep bones, aiming at elucidating the early history of domesticated sheep (Cai et al. 2007, 2011, Horsburgh 2010, Niemi et al 2013). In addition hair (including wool) from archaeological sheepskin and textile samples have been studied (Brandt et al. 2011, Olivieri et al. 2012). Most of the studies of archaeological sheep samples have focused on mitochondrial DNA, whereas Niemi et al. 2013 demonstrated the possibility to also study nuclear markers.

Though mtDNA has proven valuable for studying initial sheep domestication, its resolving power may be to low to provide information on population migrations and internal relatedness as well as to distinguish breeds.

As demonstrated by the analysis of modern sheep breeds by Tapio et al. 2010 and Kijas et al. 2012, autosomal nuclear markers are more powerful population genetic markers and provide better chances for characterising prehistoric population and mapping genetic population structure than uniparentally inherited markers such as mtDNA.

This study of ancient Danish sheep and sheep wool therefore explored if it was possible to investigate nuclear markers coding for sheep wool. In addition, the entire mitochondrial genome was also targeted in order to estimate general sample preservation and enable comparison with previously published archaeological sheep samples and modern breeds.

Wool quality

Changes in the original sheep coat were an essential change during domestication, so as to enable a wool product that can be used as fibre for textile production. Much research on wool quality in modern sheep has been carried out, as modern wool industry has had great interest in refining wool and therefore carried out many genetic studies to find genes coding for wool quality. These studies have focused, on amongst others, fibre dimension, colour, crimp and staple length, which are among the most important criteria determining wool quality (Rast-Eicher 2008: 122-126, Rast-Eicher and Bender Jørgensen 2012: 1224, Gleba 2012: 3644). Thanks to such studies, several genes coding for wool quality are known.

Wool quality inheritance

Wool quality traits as fiber diameter, length and strength have obvious funtional and visible effect on wool and the type of material that can be produced from it. The hypothesis, based on fibre messurements af wool from prehistoric textiles, is over time to see a decrease in fibre diameter and an increase in fibre length and strength. These three traits are therefore the ones in focus in the genetic study.

Visible characteristics, which are the result of genetics and environment, are called phenotypic traits. There are two types of inheritance of phenotypic traits (Sørensen et al. 2005: 166): qualitative inheritance and quantitative inheritance. Qualitative inheritance is the inheritance of phenotypic traits that are under control of major genes and vary discontinuously in a population. An example of this is blood type, which is influenced by only one gene and has only four possible outcomes: A, B, O, and AB, which are present in the population in very different amounts. Quantitative inheritance on the contrary is the inheritance of phenotypic traits that vary in degree depending upon the cumulative action of many genes and their environment, therefore it is also referred to as polygenetic inheritance. Quantitative traits vary continuously in a population. An example of this is human height, which is normal distributed. The quality of wool is influenced by many genes and environment, and is thus an example of quantitative inheritance. Therefore, studying wool quality over time, is not as simple as studying blood type, as it is nessesary to look at many genes.

Effect of genes and environment on wool quality

Refinement of traits, such as wool quality, based on quantitative inheritance, can be done by selecting animals with the best wool quality for breeding. If selecting for, for instance a narrow fibre diameter, over time, this would decrease. The speed of this process depends upon the heritability of the trait

selected for. Heritability itself is a constant, expressing how hereditary a character is. This itself represents how much of the trait is influenced by the environment as opposed to underlying genetics. When a trait's heritability is 1, it is only dependant on genes. In contrast, if it is 0, it is only dependant on the environment. Heritability can be used for calculating the expected increase in outcome by selection (Sørensen et al. 2005: 166-168). The heritability for wool quality has been documented as lying between 0,17 and 0,75 (Safari and Fogarty 2003). This directly suggests that for some trits, relatively few generations (thus ultimately time) should be required to modify the trait. This is however also dependant on, if wool is the only thing selected for. If e.g. meat weight is also priorited, the animals selected may not be the ones with the very best wool quality, and then the refinement process will take longer time (Sørensen et al. 2005: 168).

Wool quality genes studied

The wool quality genes studied here were chosen from the available studies adressing wool quality and coding for traits of main importance for selection of wool quality: fiber diameter, length and strength (Rast-Eicher 2008: 122-126, Rast-Eicher and Bender Jørgensen 2012: 1224, Gleba 2012: 3644).

Marker	**Genetic trait**	**Effect on**	**Reference**
KAP6	Keratin-associated protein	Fibre traits	Gong et al. 2011
PROP1	Combined pituitary hormone deficiency	Fibre diam.	Zeng et al. 2011
ADRB3	b3-adrenergic receptor	Staple strength	Forrest et al. 2009
KAP5-4	Keratin-associated protein	Fiber traits	Gong et al. 2010
KAP1.1, 1.3, K33	Keratin-associated proteins	Fibre traits	Itenge-Mweza et al. 2007

Table 2. Wool markers included in the study.

Material considerations

Prehistoric sheep genetics can be studied using at least two relatively common zooarchaeological sources: hair in the form of wool, and osteological material such as bones and teeth. These sources provide different opportunities. Wool textile samples, provide direct evidence of sheep used for textile production. This is not the case with sheep bone samples, as we cannot be certain about what product these were exploited for. Sheep bones on the other hand provide us with the opportunity to look at the development of sheep before sheep wool was utilised, and seem to have better chances for yielding nuclear DNA (Brandt 2010). Based on the assumption that at from a not clearly defined time point, several sheep breeds are present in Scandinavia, both sources of material are highly important for studying sheep and sheep wool development. The aim was to include both sheep wool textiles and sheep bones in the analysis and to cover the time period from the first Danish sheep around 4000 BC to the end of the Viking period (1050 AD).

Selection of textiles for DNA analysis

Previous research on DNA from wool has demonstrated that the burial environment of archaeological textiles has a large effect on their preservation of DNA (Brandt et al. 2011a, Brandt 2011). Brandt et al. for instance showed that a textile from a Danish oak log coffin did not yield amplifiable DNA (Brandt et al. 2011). Difficulties with extraction of DNA from this environment have been confirmed by recent extensive studies of DNA from human tissue from Danish oak log coffins carried out at Center for GeoGenetics under the RISE project (unpublished data, pers. comm. Morten Allentoft). These difficulties are most likely caused by the acidic environment created inside Bronze Age burial mounds (Breuning-Madsen et al. 2001: 692, Breuning-Madsen et al. 2003: 343).

Despite the succes of extracting DNA from a textile found in a Danish bogs and dated to the Pre Roman Iron Age (Brandt et al. 2011), bogs are generally not expected to preserve DNA to a degree in which it can be extracted and amplified (Hughes et al. 1986). Based on experiences from Brandt et al's study, oak coffins and acidic bogs seem to provide to worst chances for retrieving DNA from Danish archaeological textiles (Brandt et al. 2011) and therefore other contexts would be preferable.

The selection of textile samples was performed in collaboration with Senior Researcher Ulla Mannering, Centre for Textile Research and the National Museum of Denmark, through a search in the available catalogs of Danish archaeological textiles. Based on the experiences with the effects of burial environments in (Brandt et al. 2011), the archaeological context of textile samples for analysis was

carefully considered. Also the presence of dyes and mordants that can degrade DNA were considered (Brandt et al. 2011).

Despite a fantastic collection of Danish prehistoric textiles, the majority of these derive from contexts, which are not expected to yield amplifiable DNA. All preserved Danish textiles from the Early Bronze Age were unearthed from oak log coffins, which as demonstrated provide badly preserved DNA. One textile sample from Borum Eshøj (B685) which was also extracted in (Brandt et al. 2011) but yielding no result was however included, to investigate if the improved methodology (see below) gave better results.

Only a few textiles from the Late Bronze Age are preserved (Bender Jørgensen 1986: 15, 191-194), as this is a time period in which cremation graves dominate. The preserved fragments are often found in connection with bronze objects. An example of this are the wool textile fragments from Voldtofte which were found in association with a bronze urn (Thrane 1984: 27). One of the fragments from Voldtofte was tested by Brandt et al. but did not yield any DNA (Brandt et al. 2011). It is very likely that the interaction with metal ions have a degrading effect on DNA as seen for mordants (Brandt et al. 2011). This is very unfortunate, as textiles are often preserved in association with metals as they prevent micro-organism infestation (Bender Jørgensen 1986: 7, 9). Because of these low expectations for DNA preservation in textiles found in association with bronzes, fragments from the Late Bronze were not included in this study.

In the Pre Roman Iron Age, creation graves also dominate and most direct evidence of textiles come from the costumes found in peat bogs (Bender Jørgensen 1986: 26, 194, Mannering et al. 2010).

From the Roman Iron Age, more textile finds from inhumation graves have been recorded, though these are often only minor fragments. Most direct evidence of textiles from this period comes from corroded fragments preserved in association with iron objects and entire costumes from peat bogs (Bender Jørgensen 1986: 194-204, Mannering et al. 2010). A textile from Hammerum was tested by Brandt et al. but did not provide any result (Brandt et al. 2011). However, because of the value of the find, another analysis was attempted with the improved methodology. Three fragments from Lønne Hede were also tested here.

The six samples listed below made up a test to investigate DNA survival.

Site	Number	Context	Dating
Borum Eshøj	B685	Oak log coffin	1350 BC
Hammerum	1-HEM3231 x83	Inhumation grave	0-130 AD
Hammerum	2-HEN3231 x88	Inhumation grave	0-130 AD
Lønne Hede	C33202 1b nr. 4106	Inhumation grave (1969)	Early Roman Iron Age B1b
Lønne Hede	Tx 2.3 E prøve 7	Inhumation grave (grave 2)	Early Roman Iron Age B1
Lønne Hede	Tx 1.6 p.	Inhumation grave (grave 1)	Early Roman Iron Age B1

Table 3. Included textile samples. Lund Hansen sets B1a to 1-40 AD and B1b to 40-70 AD (Lund Hansen 1995: 18).

Method

For the amplification of mitochondrial and nuclear DNA, Illumina library build (Meyer and Kircher 2010) and enrichment by target-capture-sequencing was chosen (Maricic et al. 2010, see method section). This was done in order to enable amplification of shorter sequences from potentially less well preserved samples than demonstrated by Brandt et al. 2011. The capture-approach makes the amplification of specific regions more efficient as the bait (See Maricic et al. 2010) can be designed to enrich exact regions of interest.

Extraction of DNA from archaeological textiles

Extraction of DNA was performed by a more advanced protocol than the one used by Brandt et al. 2011 for ancient DNA studies on textiles. Specifically, a silica pellet extraction was chosen as it has been shown to increase the DNA yield in comparison to other extraction methods (Rohland and Hofreiter 2007). A better DNA yield leads to better chances of a large variation of sequences which is especially crucial for the capture-approach chosen, where the variety of sequences are of extreme importance. Silica pellet extraction was performed according to Orlando et al. 2011. However for textiles, the digestion buffer was replaced with the digestion buffer used in (Gilbert et al. 2004).

After extraction, samples were amplified to test DNA recovery using the 97 bp primers designed specifically for the sheep mitochondrial genome. All samples failed to yield DNA. As these initial tests of archaeological textiles did not yield promising results, focus was moved to sheep bones.

Selection of sheep bones for DNA analysis

The selection of Danish sheep bones is much larger and it was therefore possible to set up more criteria to the material. In the beginning, the idea was to perform an analysis covering prehistoric sheep from

all Danish time periods and types of settlements. This was however not possible due to the limited time frame of a Danish PhD (see Preface). As representativity is impossible to achieve in archaeological contexts anyway (see theory section), the idea was therefore instead to select a fewer number of samples from varying time periods to study 1) the applicability of the method 2) overall trends.

An initial screening was performed focussing on individuals from:

- Archaeological sites dating throughout the span from the trasition between the Mesolithic and the Neolithic to the Viking Period
- Archaeological contexts with evidence for the use of the site and the ovinoculture (settlement, cultic site, grave etc., meat, milk, textile production)
- Sites with environmental conditions which are believed to preserve DNA well
- Bones assemblages which have been examined by zooarchaeologists and present species identifications, MNI/NISP and demographic curves

The criteria for chosing a bone for analysis was first of all, that the element chosen should specificly be species identified to sheep. Species identification can be problematic between sheep and goat, as they are closely related species. Only specific elements can osteologically be identified to species level (Wigh, 2001: 37). Moreover, if more than one sample was selected from each site, it should be clear that that they derived from different individuals. This could be ensured by for instance sampling the same element from the same side, for instance right metatarsus.

The selection of bones was performed in collaboration with zooarcheologists Anne Birgitte Gotfredsen and Kristian Gregersen from the Zoological Museum, Natural History Museum of Denmark, and derive from the museum collection. Possible sites were found through a search of archaeological and zooarchaeological literature and the museum database, that holds information on all osteological finds of the museum.

As described above, preservation is an important taphonomic factor, especially in bioarchaeological studies where biomolecules may be broken down as will be discussed later. Preservation conditions were therefore an important criteria for selection. Despite apparently good preservation conditions, some sites were discarded based on the visual apperance of the bones. Bones that had a butter like consistence, lacked the surface or were very light in weight were rejected.

As part of his bone selection process, K. Gregersen evaluates the preservation conditions of the bones using simple criteria. Apart from the find conditions, these include the colour of the bone powder and

the smell that occurs by drilling, the weight of the bone, and the number of cracks (Pers. comm. K. Gregersen 20.09.2011). Even though these criteria are not confirmed, Gregersen has experienced a tendency that dark, light bones that yield a "dry" powder have low succes rates.

These samples were extracted and put through an initial PCR to test DNA preservation using the same 97 bp primers for the sheep mitochondrial genome as for describes for the textiles. 20 of the 34 extracts were succesful. The concentrations of these samples were subsequently measured with a Qubit. From this screening seven samples were selected for further analysis. These were among the ones that demonstrated the best DNA concentrations.

Ancient samples

The following ancient sheep bone samples were chosen for further analysis.

Sample no.	Locality	Parish (sogn)	District (herred)	County (amt)	Element	Museum no.	Date/calibrated 14C date BP* (95,4% probability) and dating no.	Mg of bone powder extracted
P47/2012KMG	Bulbjerg	Lild	Vester-Han	Thisted	metatarsus	ZMK 17/1913	Late Bronze Age (Müller 1919)	215 mg
P52/2012KMG	Langø	Stubberup	Bjerge	Odense	radius	ZMK 4/1928	OxA-27067 - 3514±30 ucal. BP*	230 mg
P87/2012KMG	Norsminde	Malling	Ning	Aarhus	humerus	ZMK 28/1983	OxA-27118 - 4927±34 ucal. BP*	170 mg
P89/2012KMG	Havnø	Visborg	Hindsted	Aalborg	humerus	ZMK61/1997	Uba-20320- 4848 ± 41 ucal. BP*	160 mg
P90/2012KMG	Kærup N	Benløse	Ringsted	Sorø	tibia	ZMK7/2007	Late Roman Iron Age C1b-C2	170 mg
P94/2012KMG	Skovgårde	Udby	Bårse	Præstø	Metatarsus	ZMK47/1988	Late Roman Iron Age C2	210 mg
P112/2012KMG	Storskoven	Kirke-Hvalsø	Volborg	København	tibia	ZMK46/0000	Late Roman Iron Age C2	288 mg

Table 4. Included sheep bone samples. BP =Before Present (1950). Lund Hansen sets C1b to 210/220-250/260 and C2 to 250/260-310/320 (Lund Hansen 1995: 18).

The sample from Bulbjerg comes from a Late Bronze Age settlement site in Northern Jutland. The samples from Langø, Norsminde and Havnø all come from settlement sites close to the coast. They were all believed to be of Early Neololithic date, but were recently C^{14}-dated (Sørensen 2014). The new datings placed the bone from Langø at the transition from Stone Age to Bronze Age 1920-1750 cal. BC (Sørensen 2014). Norsminde and Havnø however were however dated to respectively 3775-3647 cal. BC and 3708-3526 cal. BC placing them in the Early Neolithic period (Sørensen 2014). The last three samples from Kærup N (Grave A 3663), Skovgårde and Storskoven come from Roman Iron Age graves, in which the sheep bones were placed as food offerings (Ethelberg 2000, Gotfredsen in prep.).

Modern sample

For production of bait for target-capture sequencing (Meyer and Kircher 2010, see method section) a modern sample was collected. Fresh sheep blood[11] was collected in RNA later and extracted DNA using the DNeasy Blood and Tissue kit (Qiagen, USA) following the manufacturer's instructions. For capturing the entire mitochondria, long PCR amplification was performed using five overlapping primer sets (see table 5). We pooled purified PCR products from each sample, in equimolar amounts and fragmented the pooled long-range PCR products over 4 cycles (15 sec. on, 90 sec. off) using a Diagenode Bioruptor, to obtain 100-500 bp fragments (Maricic et al. 2010).

Primer	Position	Sequence	T_a (°C)
1F_4064 1R_8415	4064-8415	CCACAGAAGCATCAACCAAA TAATCGTACGGCAAGGGCTA	56°C
2F_8337 2R_12818	8337-12818	CACCCCACTGATCCCAATAC GTCTTGCTCGTCGTTTAGGC	56°C
3F_11007 3R_15178	11007- 15178	GCCAAACGGACCTAAAGTCA GCCTCCAATTCATGTGAGTG	56°C
4F_15084 4R_4480	15084-2865	CATCAAAGCAACGGAGCATA TACAACGTTTGGGCCTTTTC	56°C
5F_2004 5R_5464	2004-5464	CGGCCGCGGTATTCTGACCG CGGGTTGGCCTAGTTCGGCG	56°C

Table 5. Primer sets for long PCR amplification of the entire mitochondria.

Method

DNA extraction

Samples of 160-288 mg bone powder were drilled from the bones in the clean laboratory at Centre for GeoGenetics to avoid contamination.

Extraction of DNA was performed appling a new protocol compared to the one previously used for ancient DNA studies on textiles by Brandt (Brandt et al. 2011a, 2011b) to increase the DNA yield from each bone samples (Orlando et al. 2011, see above).

[11] The modern cattle sample was obtained with the kind permission of Lennart Engberg Carlsen from the slaughterhouse Anubis, Department of Basic Animal and Veterinary Sciences, Grønnegårdsvej 7, DK-1870 Frederiksberg.

Library build

We built indexed Illumina libraries for samples with the NEBNext® DNA Library Prep Master Mix Set for 454™ kit (New England BioLabs, ref: E6090), according to Protocols: NEBNext End Repair Module Protocol, with slight modifications (alternative protocol included).

The libraries were enriched for mitochondrial fragments and SNPs following the procedure described by Maricic et al. 2010. The captured library was purified through MinElute columns, amplified, and sequenced in paired-end-read mode (150 bp) on an Illumina MiSeq platform at the Danish National High-throughput DNA Sequencing Centre.

Documentation of data

Drilling, extractions and PCR setups were obtained in a physically distinct ancient DNA laboratory. Full bodysuits are worn and the lab is UV irraidated at night and has a positive pressure. Blanks were included in all extractions and amplifications.

Data analysis

Sequences retrieved from ancient material sources suffer from two main problems: the first being their mix of edogenous and exogenous sequences, the latter deriving from mainly microbial sources. The second being their often severe *post-mortem* DNA damage which shows by high nucleotide misincorporation rates (see method section) (Hofreiter et al. 2001b, Briggs et al. 2007). Amongst others, these problems are dealt with during the data analysis.

The sequences recieved from the Sequencing Center were analysed in the following way by Jose Alfredo Samaniego. Initially, the adaptors from the capture were removed with the AdapterRemoval (Lindgreen 2012). Next, sequences below 25 bp were discarded, as the propability the such a short sequence will map several places in different organisms by chance is high. Sequences containing Ns and sequences of quality below Phred 35[12] were discarded. Only the forward reads were analysed, as collapsing the perdent sequences could decrease the yield of the expected already short sequences.

Sequences were mapped to the references using BWA (http://bio-bwa.sourceforge.net/) (Li and Durbin 2009). PCR duplicate sequences and paralogs (sequences found more than one place in the genome) were filtered with Picard (http://broadinstitute.github.io/picard). The GATK tool kit (McKenna et al. 2010) was used to map the cleaned sequences and Bed tools (Quinlan and Hall 2010) was used to to meassure the coverage.

Sheep reference sequences were downloaded from NCBI (Oar_v3.1), specifically the 26 nuclear

[12] for Illumina 1.8 (L)

chromosomes (NC_019458.1-NC_019484.1) and the entire mitogenome (NC_001941.1).
The fasta files produced were used for phylogentic analysis.

Results

Authenticity

The included controls had very few reads compared to the samples and no mapping reads, demonstrating that laboratory based contamination was at a minumum.

Patterns of nucleotide misincorporations can be used as a measurement of sample age as the frequency of C to T nucleotide misincorporations towards 5′-ends of DNA fragments exhibit a strong and positive correlation with age (Sawyer et al. 2012). Due to the blunt-ending of the ancient DNA with *T4* DNA polymerase during library build, nucleotides that carry complementary and reversed versions of the 5′-ends of the opposite strands will be added. This causes apparent G to A substitutions towards the 3′-ends, which in fact reflects miscoding lesions towards the 5′-ends of the complimentary DNA strands (Briggs et al. 2007). Such nucleotide misincorporations patterns can be used to support the claim that DNA sequences are old.

We constructed Map damage profiles of our samples (see figure 23 and 24). Of these, the ones from Havnø and Storskoven demonstrated nucleotide changes from C->T in the beginning of the sequences and G->A at the end of the sequences. Because of this and the blank controls, we argue that DNA is endogenous to the samples.

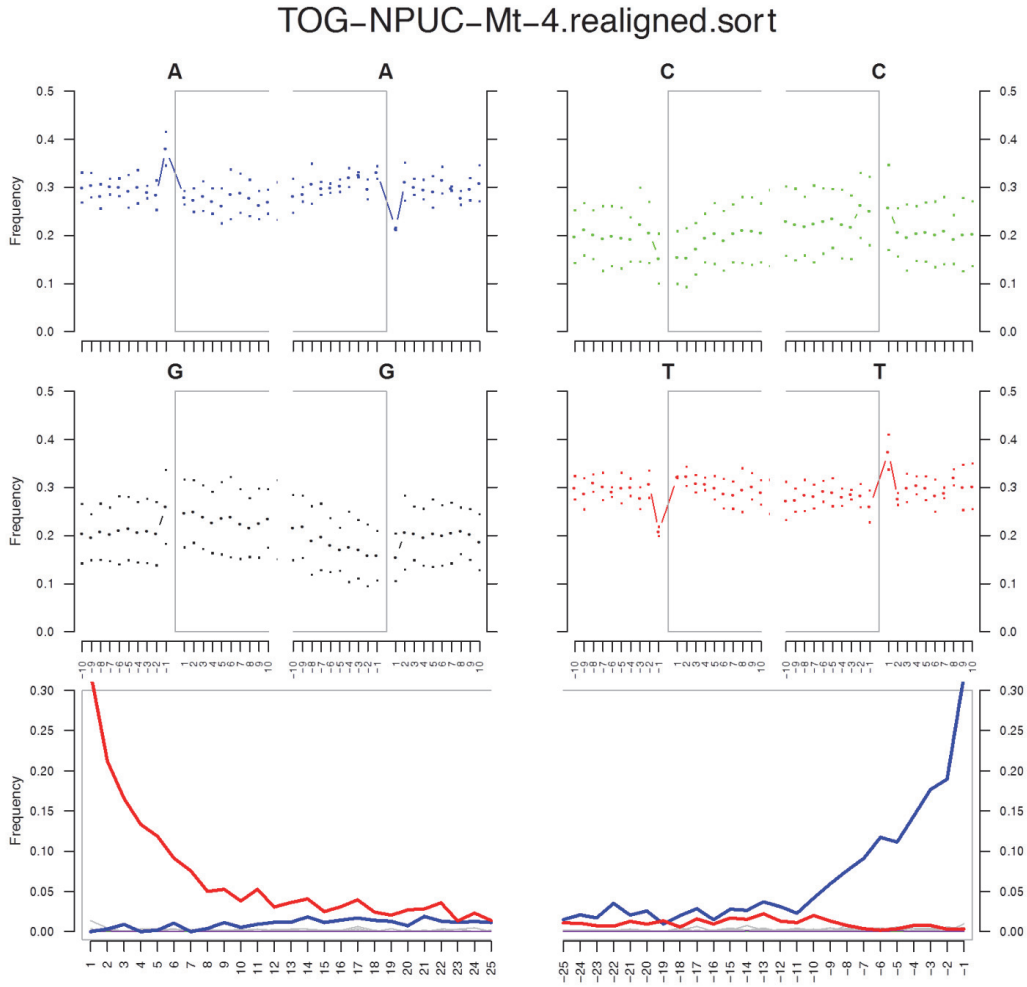

Figure 23. Nucleotide misincorporation pattern for the sample from Havnø (TOG-NPUC-Mt-4). Base frequency along the fragment.

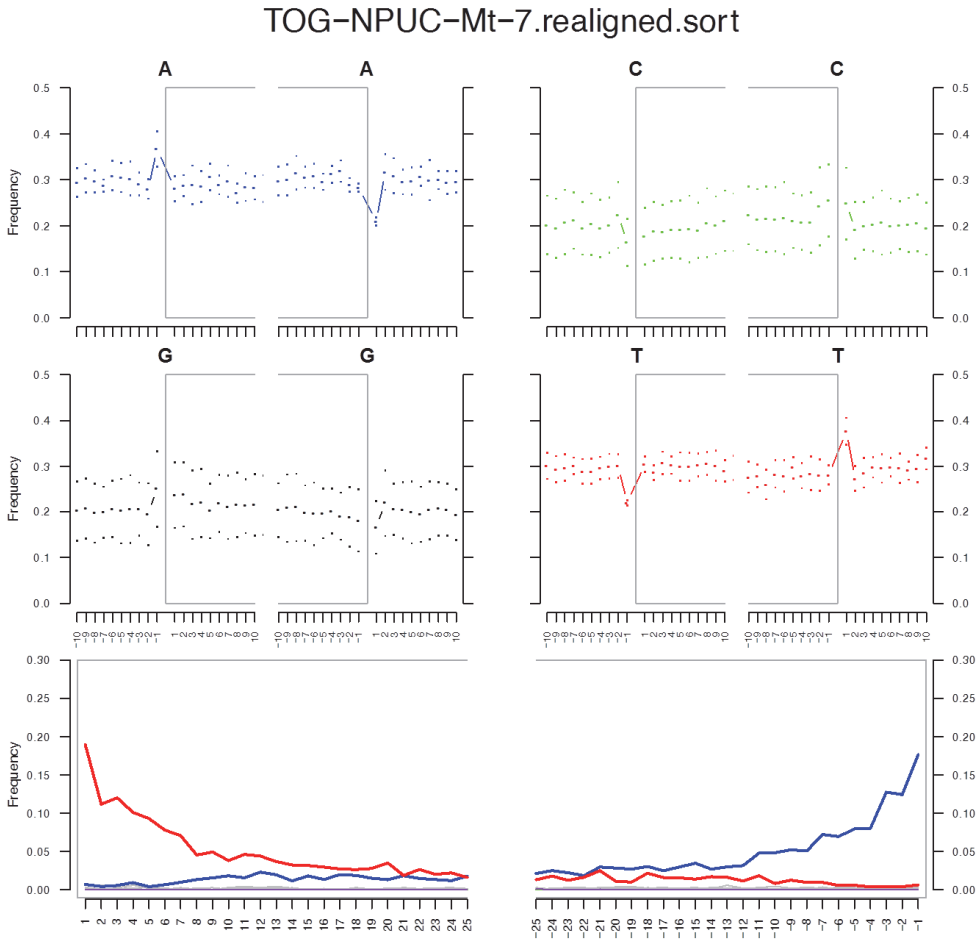

Figure 24. Nucleotide misincorporation pattern for the sample from Storskoven (TOG-NPUC-Mt-7). Base frequency along the fragment.

We generated two almost complete mitogenomes from two ancient sheep bone samples. The mitochondria of sample 4, Havnø, was covered by 93,68% with a mean coverage of 7,14 times, and the mitochondria of sample 7, Storskoven, was covered by 99,58% with mean coverage of 18,46%. The uncovered regions are recorded in Table S1 in the Appendix. For the other samples, the mitochondria was less well covered.

Ovis	Sample	Raw reads	Trimmed reads	Mapped reads	After RmDup	Unique reads	% of MT covered	Mean coverage in MT	Mean coverage in covered regions
MT	Bulbjerg	2839213	2770751	38	29	29	11.2061%	0.118079X	1.05371X
	Langø	4482448	4444984	28	25	25	6.24097%	0.077636X	1.24397X
	Norsminde	1683155	1627231	21	19	19	4.51974%	0.0484473X	1.0719X
	Havnø	2662117	2580389	3345	2335	2334	93.6808%	7.14065X	7.62232X
	Kærup N	2986550	2893621	72	69	69	17.0558%	0.204321X	1.19795X
	Skovgårde	3065575	3022172	321	228	228	55.5188%	0.876986X	1.57962X
	Storskoven	1665449	1640227	6476	4760	4760	99.5787%	18.4582X	18.5363X
	Blank	339	311	0	0	0	0	0	0
	Undetermined	1015591	978043	428	413	413	67.4952%	1.39733X	2.07026X
Ovis	**Sample**	**Raw reads**	**Trimmed reads**	**Mapped reads**	**After RmDup**	**Unique reads**	**% of regions covered**	**Mean coverage in regions**	**Mean coverage in covered regions**
Nuclear regions	Bulbjerg	2555488	2512569	0	0	0	0%	0X	0X
	Langø	1817195	1794390	3	2	2	3,00%	0.0299685X	1X
	Norsminde	2208690	2152787	2	2	2	14,96%	0.149554X	1X
	Havnø	2630432	2563500	17	15	15	14,25%	0.187379X	1.31456X
	Kærup N	2391244	2336087	8	5	5	6,76%	0.0676145X	1X
	Skovgårde	2540346	2483199	0	0	0	0%	0X	0X
	Storskoven	1790107	1786134	75	49	49	42,82%	0.807362X	1.88559X
	Blank	2293	2226	0	0	0	0%	0X	0X
	Undetermined	2787845	2710896	1	1	1	6,46%	0.0646095X	1X

Table 6. Number of reads and coverage of the captured samples for nuclear and mtDNA. 4=Havnø, 7=Storskoven

Very few nuclear sequences mapped to the reference (see Table 6). Sample 4 and 7 display most mapping reads, whereas no nuclear reads mapped for sample 1 and 6 mapped. For samples with mapping nuclear sequences, the ratio of nuclear to mitochondrial mapping reads is around 1:10 to 1:150. This difference in ratio is lower than the proposed ratio of nuclear DNA to mtDNA, which is 1000-10000 of mtDNA to nuDNA copies in every cell. This indicates, that there is no particular problem with preservation of nuclear (as opposed to mitochondrial) DNA in the samples, but that the DNA preservation in the samples was generally poor.

This observation is supported by the low amount of endogenous DNA (trimmed reads/mapped reads) which range between 0,00001-0,003%. Though endogenous DNA content of sequenced Illumina libraries of archaeological bones has been demonstrated to vary greatly, the endogenous DNA content on samples that have been successful in other DNA capture experiments has been at least 100-1000 times more endogenous than the samples presented here (Bennett et al. 2014, Sandoval-Velasco et al. in review). This demonstrates that the DNA preservation of the included samples has not been optimal. The reasons for this will be discussed below.

The ancient sheep samples

The two ancient Danish sheep samples were mapped against the sheep reference sequence, NC001941. The polymorphisms of the ancient Danish sheep mitogenomes compared to the reference are reported in Table 7.

To describe the relationship between the two ancient Danish sheep specimens and modern domestic sheep, two phylogenetic trees were constructed, one based on entire sheep mitogenomes, and one based on the complete cytochrome b sequence, as cytochrome b has been more intensively studied for sheep phylogeny than the entire mitochondria. For the entire mitogenome (16616 bp for the sheep reference NC001941) we retrieved 45 published complete mitochondrial sequences from GeneBank (see Table S2). For the complete cytochrome b sequence (1140 bp for the sheep reference NC001941 from position 14159-15298) we retrieved 159 sequences from GeneBank (120 and the cytochrome sequences extracted from the 45 complete mitogenomes) (see Table S3).

Nucleotide position	Reference sequence NC_001941.1	Havnø	Storskoven
281	T		C
1115	T	C	
1731 ins		no info	C
3036	T	C	
3544	T	A	A
6615	G	A	A
7500	C	A	A
8264	G	C	C
8652	C	T	
9375	A	G	G
10862	G	A	
11668	G	A	A
12539	G	C	C
12571	G	C	C
13199	A	G	G
13813	G	C	C
14055	T	C	C
15721	T	no info	C
15924	A	G	
15995	C		T
16128	C	T	T

Table 7. Danish ancient sheep mitogenome nucleotide polymorphisms relative to *Ovis aries* reference sequence (NC001941).

The complete mitochondrial and cytochrome b sequences obtained from the ancient sheep samples were aligned with the retrieved sequences available in GenBank (See Table S2 and S3), and an outgroup (the goat (*Capra hircus*) reference sequence NC005044) using the ClustalW software.

A phylogenetic tree of the aligned sequences was constructed using PhyML 20131022 software (Guindon and Gascuel 2003) applying the ML (Maximum Likelihood) GTR (Generalised Time Reversible) + G+I substitution models.

Both the phylogenetic trees shown in figure 25 and 26 (in full scale see fig. S1 and S2) place the two ancient Danish sheep samples within haplogroup B, that represents the main haplogroup in modern European sheep. Both of the constructed phylogenetic trees represent all the five haplogroups defined in modern sheep (see Table S2 and S3)

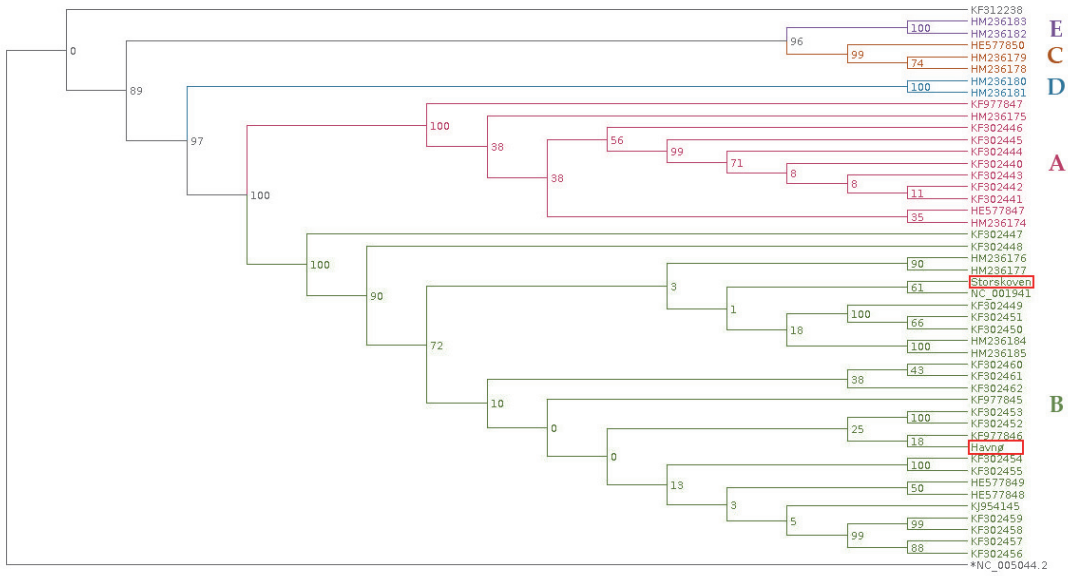

Figure 25. Cladogram of modern sheep based on 45 modern, two ancient complete mitocondrial sequences, and an outgroup, *Capra hircus*.

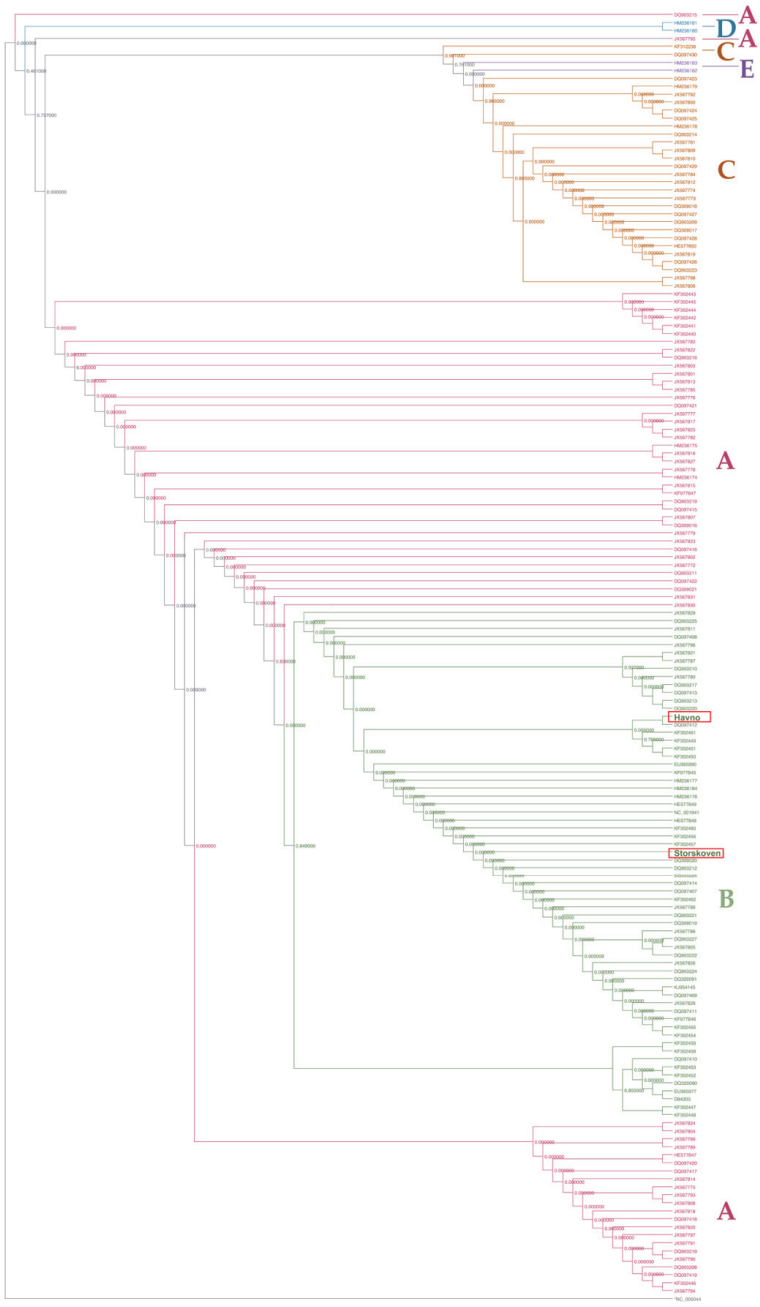

Figure 26. Cladogram of modern sheep based on 159 modern, two ancient complete cytochrome b sequences, and an outgroup, *Capra hircus*.

Phylogentic structure of sheep

The two ancient samples cluster slightly differently for the complete mitochondria and the cytochrome b to the 45 samples that were included in both phylogenetic trees. For cytochrome b, a motif of position 309: C and 495: G and no other differing nucleotide positions characterises many of the samples in haplogroup B, including Havnø and Storskoven. Based on this, the two ancient samples seem to represent a haplotype common in modern breeds.

For the complete mitochondria, Havnø and Storskoven differs from each other at position 281, 1115, 8652, 10862, 15924, and 15795. Both of the ancient samples have unique haplotypes based on the mitochondrial DNA. This way, our data this way demonstrates how the complete mitochondrial sequence provides further resolution in the phylogenetic relationship compared to partial sequences. Complete mitochondrial genomes often provide increased information when compared to short mtDNA sequences (Paijmans et al. 2013). In our case, the complete mitochondria provided a refined phylogenetic resolution.

While haplogroup C, D, and E are less common and more geographically restricted to specific regions, the haplogroups A and B are frequent. Haplogroup B is found frequently in European breeds (Wood and Phua 1996, Hiendleder et al. 1998). It is notable that a sheep skin sample from the ancient Iceman Ötzis clothing was also reported as falling in haplogroup B based on a 2,429 bp fragment of the a part of the control region, the tRNAPhe, a part of the 12S rRNA gene, and the whole cytochrome B gene. The sequence is however not publicly available (Olivieri et al. 2012), and could thus not be included in the analysis.

Both haplotype A and B were identified in a sample set of 27 ancient and historical Finnish samples based on a 523 bp fragment of the mtDNA D-loop region (Niemi et al. 2013).

The only published article from the group at the ZBSA working with sheep wool development (Zentrum für Baltische und Skandinavische Archäologie at Schloss Gottorp, northern Germany) describes that they extracted and sequenced an undefined part of the cytochrome b gene of Danish and German prehistoric sheep. Based on their sample set haplogroup B seems to become more frequent after the Common Era (Nikulina 2012).

Haplotype B therefore also seems frequent in however still studies of ancient European sheep. The resolution is however low in the often small fragments studied. Moreover, the regions studied have not been consistent and are therefore hard to compare with. If possible, future studies should focus on full mitochondrial genomes rather than shorter sequences as this provides a higher resolution that could clarify haplotypes further.

Discussion

The quality of the textile samples

None of the included ancient textile samples (see Table 3) yielded amplifiable DNA. As previously demonstrated, the environment in the burial mound Borum Eshøj C is wet and characterised by low pH values (Brandt et al. 2011). It is thus, not surprising that no DNA survived in this sample. It was however the hope that the optimized extraction protocol compared to the one used by Brandt et al. 2011 could overcome these issues, but this was unfortunately not the case. As earlier metioned, the difficulties in obtaining DNA from the Bronze Age oak coffins of Danish burial mounds have also been reported on human hair and bone by researchers in the RISE project (unpublished data, pers. comm. Morten Allentoft).

Based on the experiences of Brandt et al. 2011, five samples from inhumation graves were chosen for analysis as these were considered to provide better chances for DNA recovery than oak coffin graves and acidic peat bogs. This was not an easy task as most of the well preserved ancient Danish textiles samples come from those two types of environments.

Despite the effort to select samples from other enviroments, the samples did not yield amplifiable DNA and it is speculated if the older age compared to the succesful samples from Brandt et al. 2011[13] could explain this. Therefore it seems that samples of younger age as for instance samples from Viking period, medieval or historical times, and from cold environments such as those found in Greenland, or alkaline burial environments present the best opportunities for future research (Brandt et al. 2011). Recovery of nuclear DNA from wool has to my knowledge not been demonstrated so far. The difficulties of retrieving nuclear DNA is caused by the numerical advantage to mtDNA. It therefore seems that recovering nuclear DNA from wool would require extraordinary conditions.

The quality of the sheep bone samples.

Two of the seven tested ancient sheep samples provided almost complete mitogenomes. The last five samples yielded varying, but very poorly, covered mitogenomes. All samples yielded few or no mapping reads of nuclear DNA. Although this numerically reflects the natural frequency of nuclear DNA to mitochondrial DNA sequences, the difficulty of extracting and sequencing nuclear markers in ancient and even historical sheep samples was also demonstrated by Niemi et al. (2013). Overall it seems clear that the low number of mapping reads reflects that the DNA of the samples is highly degraded, which is also demonstrated by the short read length.

[13] except for the sample over 2000 year old sample from Krogens Mølle Mose, a bog which turned out to have an extraordinary alkaline environment

The degradation of endogenous DNA has been suggested to be linked to factors such as sample burial and exposure temperature, the time before sedimentation, chemical properties of the burial environment, pH value, storage, and the presence or absence of oxygen, water, ionic radiation, and microorganisms (Allentoft et al. 2012, Elsner et al. 2014).
Several methods have been used as indicators of DNA preservation. These methods include histology (Guarino et al. 2000), amino acid racemization (Poinar et al. 1996) and thermal age (Smith et al. 2001).
All of these have however have however been shown to have an inconsistent correlation with DNA preservation (Haynes et al. 2002, Gilbert et al. 2006, Collins et al. 2009, Schwarz et al. 2009, Hoke et al. 2011) though there is general agreement, that temperature is one of the most important factors for DNA preservation. The analysis of palaeolithic horse remains from Switzerland by Elsner et al. demonstrated that the burial environment was the most important factor for DNA preservation over age and storage time (Elsner et al. 2014). On the contrary, Allentoft et al. 2012 demonstrate differing DNA preservation in seemingly similar environments (Allentoft et al. 2012).
Age has however been shown to have a predictable correlation with DNA preservation (Allentoft et al. 2012).

In the included material, sample 4, Havnø, from an Early Neolithic settlement site, which provided an almost complete mitochondria, was the oldest sample included in the sample set. Sample 1-3 also derive from settlement sites, and though two of these are considerable younger than sample 4, they were of inferior quality.
Sample 5, 6 and 7 all derive from graves where parts of sheep were laid down as food offerings. They are contemporary and the context is similar. However, these samples performed quite differently. Sample 7 provided an almost complete mitogenome, whereas sample 5 had the lowest content of endogenous DNA, though it was the most recently excavated sample of all the included samples.

Based on the small set of samples included, younger samples did not seem to perform better than older samples. Moreover, the burial environment of the samples did not seem to have any correlation with their performance. However, factors such as the time before sedimentation, varying pH, and the prehistoric presence or absence of oxygen and water are unknown. Such factors could affect the preservation of DNA in seemingly similar burial environments. However, many factors are in play, which is probably the reason why a correlation between single factors and performance is hard to demonstrate.

A good knowledge and systematic testing of DNA preservation in different local burial contexts could provide information of the site types that are worth targeting.
For instance, Danish environments as bogs and oak coffin graves have been demonstrated to provide poor chances of retrieving DNA (Brandt et al. 2011, pers. comm. Morten Allentoft). Such information is particularly valuable when larger sample sets are tested. This is less of a problem in studies investigating only one sample, in which case it is possible to pick out an extraordinary environment and optimise the protocol specifically.

Future sampling and sample selection strategy

The seven samples included in this study were initially selected based on their higher concentrations compared to other samples. However, DNA extracted from archaeological bone samples almost always contain a mixture of endogenous and exogenous DNA. Exogenous DNA can derive from modern sources such as laboratory reagents and handling or ancient sources such as organisms involved in the degradation of tissues or present in the burial environment. The frequency of endogenous to exogenous DNA varies, however, based on amongst others the samples history including burial context and handling, target species and the methodology chosen (Wales et al. 2012). This kind of contamination is a considerable problem when using second generation sequencing shotgun techniques, as primers are not target and species specific, and much of the data can be irrelevant to the sample. As second generation sequencing is both labour and economically demanding, it is ideal to get an idea of the ratio between endogenous and exogenous DNA before choosing samples for sequencing and to choose the ones with the highest frequency of endogenous DNA. So far, samples have been chosen somewhat blindly. This is also the case in this study in which the samples were chosen based on the concentration of DNA in the extracts measured on a Qubit. However, the chosen libraries may not have been the ones containing the most sheep DNA, as much of the DNA could have derived from bacteria. At least in the context of plant remains, Wales et al. (2012) developed a real-time quantitative PCR assay (qPCR) to predict the relative amount of sample and 'contaminant' or exogenous DNA. Their study demonstrated that a qPCR test assay on preferably ancient DNA libraries is a valuable tool in screening for samples with high frequencies of endogenous DNA (Wales et al. 2012). This is an important finding in cases where multiple samples able to provide equal information are available and could be a sample strategy worth applying to Danish sheep bone samples in the future if it turns out to work equally well on animal remains.
The low number of mapping reads in the included study could also be explained by the performance of the capture method itself. Samples with a high proportion of endogenous DNA seem to perform better

in capture compared to samples with low starting amounts of endogenous DNA. This is measured by the number of non-clonal mapping reads of the total number of uniquely mapping reads between capture and shotgun experiments. Modern human DNA has been reported to be capture enriched approximately 400 fold (Tewhey et al. 2009) and respectively 3640 to 80,400-fold (Briggs et al. 2009) and approximately 190,000-fold target enrichment for Neanderthal (Burbano et al. 2010), though this ratio includes clones. On the contrary ancient maize samples were enriched by in average 4-29 fold (Ávila-Arcos et al. 2011). Though we do not have shotgun data to compare with here, the capture experiment demonstrated a low amount of endogenous DNA. Based on the trends demonstrated above, the capture enrichment in this experiment may not have been optimal.

Future methods

In this experiment, the performance of target capture sequencing was most likely challenged by the low input of endogenous DNA of the libraries. The amount of endogenous DNA could potentially be increased by improved aDNA extraction and library preparation methods.

Though the silica pellet extraction applied here (also termed silica-in-solution/SIS) has been demonstrated to be superior to other extraction methods with regards to the yield of short molecules and the variety of sequences (Rohland and Hofreiter 2007, Sandoval-Velasco et al. in review) new optimized methods provide even shorter fragments (30-50 bp) of DNA in the future (Dabney et al. 2013). This could make studies of more degraded samples possible.

With regards to library preparation, a new method for ssDNA library preparation was published recently (Gansuage and Meyer 2013). ssDNA libraries have been argued to yield higher endogenous DNA percentage post sequencing compared to dsDNA libraries, as ssDNA library preparation retains small, damaged DNA fragments that would have been lost in dsDNA library preparation. For instance this method enabled the reconstruction of a high-coverage ancient genome from Denisovan, an extinct relative of Neandertals (Meyer et al. 2012). Sandoval-Velasco et al. (in review) were however unable to show a consistent difference in the performance of DNA from ssDNA or dsDNA with regards to their yields of endogenous DNA. Both Sandoval-Velasco et al. and Bennett et al. demonstrate that ssDNA library preparation has the greatest advantage of yielding more endogenous DNA on samples with an initially low content of endogenous DNA compared to samples with more endogenous DNA to start with, as for instance younger samples (Bennett et al. 2014, Sandoval-Velasco et al. in review). The studies that provide the most promising results with regards to ssDNA libraries have been performed on a single sample (Meyer et al. 2012) and a set of samples of generally low quality (Bennett et al. 2014). These promising results may not apply to a wider set of samples as demonstrated by Sandoval-Velasco

et al. who also demonstrated that ssDNA libraries perform worse than dsDNA libraries in capture, because of their higher content of short sequences (Sandoval-Velasco et al. in review). Therefore, although ssDNA library builds present some encouraging results for samples with low content of endogenous DNA, it may not work equally well on all qualities of samples and is challenged when used in combination with capture. Therefore, if ssDNA libraries should be used for capture, it would be optimal if the capture could be optimized to retain more smaller fragments.

Implications of the phylogenetic positions of the ancient Danish sheep

As previously mentioned, analysis of modern sheep has demonstrated that autosomal nuclear markers are more powerful population genetic markers and provide better chances for characterising prehistoric population than mitochondrial DNA (Tapio et al. 2010 and Kijas et al. 2012).

Because of the poor preservation of endogenous DNA, the analysis was not able to resolve the question of wool development and the possible local development or immigration of new sheep types.

Nuclear DNA therefore seems to be needed in order to resolve the types of questions asked in this chapter. However, mitochondrial DNA could still be a useful marker in the future. Both regarding estimation of general DNA preservation in the samples, and moreover, also the distribution of mitochondrial haplogroups could have been less mixed in prehistoric sheep populations than today and display a prehistoric pattern.

Conclusion of DNA study

The seven ancient sheep samples generally yielded a low number of mapping reads, which must reflect a low content of endogenous DNA in the samples. Despite the low number of mapping reads, two samples (Havnø and Storskoven) provided an almost complete mitochondrial genome. The nuclear markers on the other hand yielded very few or no mapping reads, which simply reflects the numerical difference of nuclear to mitochondrial DNA in the cell.

The low number of mapping reads may also be explained by the capture experiment in itself, as capture has been demonstrated to enrich samples with an initial high amount of endogenous DNA more than samples with a low starting amount of endogenous DNA.

The two mitochondrial genomes placed both of the ancient Danish sheep in haplogroup B. The analysis was consistent with the expectation that the phylogenetic tree for the complete mitochondrial genome provides further resolution than the cytochrome B sequence. Despite this higher resolution, mitochondrial genomes may not be able to resolve questions of immigration and emergence of sheep

breeds, though an increase in available comparison material of complete mitochondrial genomes could enlighten if the prehistoric distribution of haplogroups was more split up than is the case today. Nevertheless, nuclear markers seem to provide the best options for resolving population genetic questions. The study of these would first of all require samples of good quality, but also the optimization of DNA extraction protocols and the building of ssDNA libraries could improve the yield of endogenous DNA.

Wool was a complicated trait to study as it is encoded for by many genes, therefore less complicated characteristics could be looked at in future studies. Even the development of genes for meat yield could provide information relevant for studies of wool qualities as in provides information on the divergence of breeds and breeding after particular products.

Future studies could also focus on optimizing the sampling strategy. This could include 1) development and application of a more detailed knowledge on local burial contexts and the preservation of DNA in these 2) qPCR assays of extracts and libraries, which will reveal if a samples DNA concentration illustrates endogenous or exogenous DNA.

Conclusion of the development of Danish prehistoric sheep and sheep wool

Judging from the so far earliest finds of loom weights, the changes in jewellery style, the introduction of the dress pin, as well as the increased contacts with the Únětice culture in which changes in sheep herding and an early find of wool indicates that wool was utilised as a textile fibre, and that changes in the costume and its production take place in the Danish Late Neolithic Period (2400-1700 BC). It is very likely that this is linked to the introduction of a new fibre: sheep wool and that textiles of wool could have been present in Denmark in Late Neolithic Period before the first actual finds in the Early Bronze Age period I (1700-1500 BC).

The sheep wool must have undergone changes during the Late Neolithic period or Early Bronze Age to reach a quality that could be used for textile production. Based on the frequency, kill-off-patterns and size of Danish sheep, wool production does not seem to be common in Denmark until the Pre Roman Iron Age (Nyegaard 1998).

Measurements of the fibre diameter in Danish textiles demonstrate that the width of the uninterrupted range does not change significantly in the Bronze and Pre Roman Iron Age (1700-500 BC and 500-1 BC), but it does do so in the Roman Iron Age (1-400 AD). Also the appearance of shears at the end of the Pre Roman Iron Age indicates changes in the fleece towards the common Era. These changes coincide with a generally increased contact with continental Europe in which fibre analysis and written sources indicate that different sheep types were already present in the first millennium BC.

Due to low amounts of endogenous DNA in the studied bone and textile samples, the DNA study was not able to provide sufficient data to add new information to the development of Danish sheep and their wool. Based on the conclusions of DNA analysis, this will hopefully be the case in the future with an enhanced focus on sample selection and methodological improvements.

Considerations and future studies

This study has led to several considerations of methodological and theoretical considerations that have implications for future studies.

The first wool textiles recovered in Denmark derive from the oak coffins dated from the Early Bronze Age period I (1700-1500 BC) to period III (1300-1100 BC). These burials are argued to represent the very top of the society because of their content of prestige objects.

Based on the frequency, kill-off-patterns and size of Danish sheep, Nyegaard for instance argues that wool production was not common in Denmark until the Pre Roman Iron Age and that only the elite wore woollen textiles, whereas large parts of the population must have worn skin clothing. Also the number of loom weights does not reflect a large production of textiles, though other types of looms not using loom weights could have been used (Nyegaard 1993: 102).

Despite this, a number of textiles dating to the Early Bronze Age has been recovered (Bender Jørgensen 1986). This opens up for the possiblity that these were not (all) produced of local wool, as Frei's analyses of textiles from later time periods have demonstrated. Her analysis of the textiles from the Early Bronze Age oak coffin from Egtved will enlighten this further. The import of textiles or wool in large scale would not be surprising compared to the amount of metal imported and for instance the amount of swords recovered from the Bronze Age (pers. comm. Flemming Kaul). Though the wool was not local, it could, however, still have been processed locally, which Mannering indicates to be most likely based on the characteristic and distinct fabricts found in Scandinavia (Mannering in prep.b).

Contrary to the idea of imported wool, the wool found in Central European textiles is coarser than the wool found in Scandinavian textiles. This indicates that the sheep delivering the wool for these textiles were of a different type (Rast-Eicher and Jørgensen 2013). The obvious solution to this would be that the textiles of each region derived from local breeds that were of different type. It may, however, not be as simple as this, which the contradicting evidence above indicates. This also has implications for the material chosen for the present and future analyses.

Originally the plan for addressing the possiblity of import of wool or textiles was to analyse sheep bones and textiles from the same settlements. The objective was to investigate if the DNA from wool

and bones corresponded to this or if it was so different that wool could have been imported. Also, in order to address the possibility of the presence of different types of sheep or breeds, samples from different contexts were selected to investigate if these differed enough to support the claim that different sheep types existed.

However, analyses of textiles and bones from the same sites proved difficult as only very few sites have both materials and even more so as none of the included textile samples were succesful in the DNA analysis. It therefore seems that the possibility of import must be tackled differently in the future. Though textiles from contexts with good environment for DNA preservation may prove successful in the future, the analysis of strontium isotopes will probably be more successful and as described earlier, strontium isotope analysis has already provided invaluable results documenting the provenance of both textiles and bones.

Fibre analysis has also been demonstrated to be a very valuable tool to explore differences between wool types. The fibre analysis is, however, limited to the time periods with preserved textiles.

The analysis of DNA is on the other hand applicable to sheep bones as well. In contrast to strontium isotope analysis, DNA cannot reveal the provenance of the sample itself, but rather its broader relationship to other populations that may be geographically linked, population diversity and specific genetic traits. For some genetic traits, as for instance wool, which is encoded for by many genes, DNA analysis cannot directly identify the phenotype of the wool. It can, however, provide an overview of the frequencies of wool coding genes and their changes over time. Here the fibre analysis of textiles provide the best answers.

Strontium isotope analysis, the analysis of ancient DNA, and fibre analysis provide different types of information which are all important in the investigation of the development of sheep wool. Therefore, the combination of these analyses is the optimal solution to continue the investigation of wool development, and ideally more than one analysis should be applied to each sample.

The results of these analyses are, however, only meaningful when they are put into an archaeological context. As demonstrated in the beginning of this chapter, the context and archaeological material provides crucial and important information to support or contradict scientific results and is essential for their correct interpretation.

Introduction

Denmark has a large collection of prehistoric skin objects from peat bogs, comprising more than 68 well-preserved items, dating from approximately 920 BC to AD 775 (Hald 1980, Schmidt et al. 2011, Schmidt and Mannering forthcoming). The formation of bogs is mainly dependant humidity and temperature. Optimal circumstances for bog formation are found in several parts of the world, but not in all - for instance not in dry climates. In the northern hemispere, North and Northwestern Europe is one of the areas where bogs are most common (Fisher 2007: 58-63, Sanden 1996). Probably mainly because of their content of sphagnum (peat moss), peat bogs are excellent environments for the preservation of organic material. First, decaying sphagnum reacts with calcium and nitrate from organic materials, where these would otherwise be object for microorganisms and thus sphagnum prevents degradation of organic materials. Second, it catalyses tanning of skins (Fisher 2007: 58-63, Ravn 2010, Sanden 1996). Because of these two processes, North European bogs have preserved historic and prehistoric organic materials as the Danish skin objects.

In the analysis of these prehistoric skin objects, species identification is a crucial aspect, as the skin used and its processing have implications for its functionality, appearance, use, and perhaps non-verbal form of communication. Furthermore, this provides information on animal exploitation (Mannering and Gleba forthcoming, Schmidt and Mannering forthcoming).
Microscopic species identification of ancient skin and hair has been demonstrated to be difficult (Scmidt et al. 2011). Moreover, recovery of DNA from acidic peat bog environments has been shown to be highly problematic (Hughes et al. 1986, Schmidt et al. 2011). Therefore, this part of the dissertation aims at finding a reliable and reproducable method for species identification of prehistoric skins from bogs.

Bog bodies and skin garments from Danish peat bogs

Bodies deposited in bogs are a phenomenon in Denmark from the Stone Age to the Medieval Period (Asingh and Lynnerup 2007: 291, Fisher 2007: 74). The Danish National Research Foundation's Centre for Textile Research (CTR) has focused on the Danish prehistoric finds of skins and textiles in bogs (i.e. excluding the medieval finds). The majority of these finds of skin garments and textiles occur on the Jutland Peninsula (Hald 1980, Mannering and Gleba forthcoming). ^{14}C datings of these selected finds place the clear majority of them in the Pre-Roman Iron Age and the beginning of the Roman Iron Age (Mannering et al. 2010). The types of garments deposited include hats, capes, hooded capes, shoes,

bags, straps and strings, a loincloth and a tunic (Hald 1980, Schmidt and Mannering forthcoming). The skin objects included in the present study belong to the largest group of articles of clothing: the capes. The number of capes acknowledged differs between publications as some skin fragments have been difficult to determine as capes with certainty (Hald 1980). The latest recording by Schmidt comprises 21 capes, three hooded capes, and a tunic (Schmidt and Mannering forthcoming). The group of capes date to the Danish Iron Age, from the transition to the Bronze Age around 500 BC to AD 550[14] (Ebbesen 2009: 42, Mannering et al. 2010: 264-265, see chronology in Table 1).

Hald divided the skin capes into three types, A, B, and C (Hald 1980: 313-17, see Fig. 27). Type A is characterised by being symmetrical and having a collar or facing at the upper edge. This type must have rested on both shoulders when worn, and was closed in front on the chest (Hald 1980: 313). Types B and C are characterised by their assymmetrical shape. Type B is distinguishued by having a collar or facing at the upper edge, whereas type C lacks this edging. Both types B and C were closed at the shoulder leaving an arm free (Hald 1980: 316-17).

The capes were probably worn singly or double as demonstrated by both the Undelev Man (Hald 1980: 69) and the Huldremose Woman (Mannering 2010) who wore two capes, of which the inner cape had the fur facing inwards and the outer cape had the fur facing outwards. With the fur facing inwards on the inner cape and the fur facing outwards on the outer cape, the capes would constitute excellent protection against cold weather as seen in inner and outer parkas in the Arctic (Hald 1980: 316-317). The inner cape would keep warm whereas the outer cape would repel water. Several bog bodies were buried with two or multiple capes and Hald suggests that, capes with slanting necklines reverse against each other (indicating they should be closed on opposite shoulders), and were actually worn as inner and outer capes, thus fitting together and closing over the same shoulder, leaving one arm free (Hald 1980: 316-17).

[14] However, two of the finds, Hørby Mose and Årestrup By (number 4 and 6 in the catalogue in Ebbesen 2009) are undated.

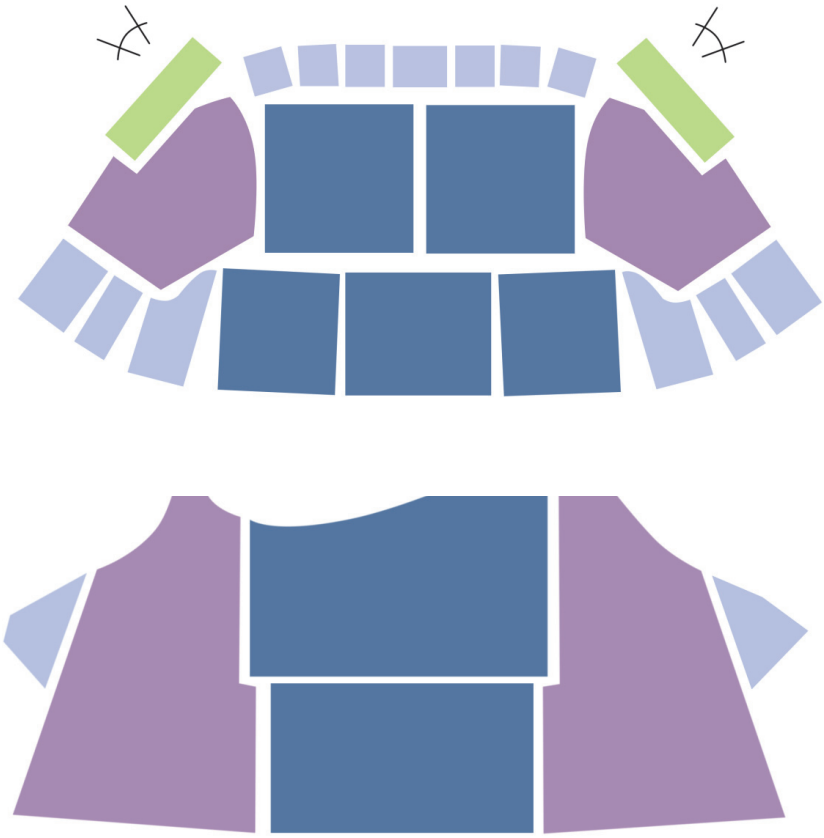

Figure 27. Design of Danish Iron Age skin capes of type A (top) and B (bottom).

The history of species identification in Denmark

Species identification has a long history in Danish archaeology. Traditionally, identifications were performed by visual inspection of the skins and by microscopy of their associated hairs. In one of the earliest studies, reported in 1836[15], the skin cape from Haraldskær was determined as sealskin by comparison to the Artic collection at the National Museum of Denmark (Oldsags-commiteen 1836-37: 163-64). In the same study, microscopic analysis was carried out on the textiles from Haraldskær by a

[15] Probably by the later director of the National Museum of Denmark Christian Jürgensen Thomsen.

specialist from the Polytechnisk Læreanstalt (today the Technical University of Denmark), who determined the fibres as sheep wool. In another early study from 1891, Bille Gram reports of the determination of hairs from skins, textiles and humans from the Danish Bronze Age oak coffins by microscopy and comparison to reference material. Interestingly, he identifies the textile fibres as a mix of sheep and deer hair (Gram 1891). This result was revisited in 1939 by Carl Marinus Steenberg who investigated the Skrydstryp textiles and demonstrated that, the coarse hairs did not derive from deer, but from primitive sheep breeds (Steenberg 1939). The species identifications of Danish skin finds referred to until recently derive from the first collected publication of the bog skin finds, Margrethe Hald's publication of the Danish collection of skins and textiles (Hald 1950, 1980). In Hald's publication, Christian Overgaard Nielsen identified the species of the skins through microscopy (Hald 1950: 140, Hald 1980: 138-39). In 1986, Lise Bender Jørgensen and Klaus Ebbesen also had a few fibre analyses made by Harry M. Appleyard, Halifax, United Kingdom which were published in Ebbesen 2006. Since 2006, a new CTR-initiated investigation of the skins from the peat bogs has, however, produced many new results.

State-of-the-art in species identification of skins

This species identification study is part of CTR's DTC research program (Danish Textiles and Costumes from the Early Iron Age). The overall aim of DTC was to document Early Iron Age costumes found in Denmark in a new and systematic way with scientific methods. As part of this, a project aiming at providing new animal species identifications of Early Iron Age skin clothes from the Danish peat bogs was initiated in 2006 in collaboration with The National Museum of Denmark and the Centre for GeoGenetics. The first results which surprisingly showed disagreements between microscopic species identification and species identification by MS-based peptide sequencing were published in Schmidt et al. 2011. In 2012, project leader Ulla Mannering received funding from The Ministry of Culture (Kulturministeriets forskningspulje, KUF) which made it possible to continue the 2006 species identification project.

The purpose of the species identifications was to investigate whether the animals used were domesticated or wild, whether several species were used for the same cape, and to identify the number of animals used for each cape. This would provide information on the type and size of animals a household would need for the production of skin clothes and the subsequent processes needed to transform skins into skin clothing.

Traditional species identification by microscopy is challenged by the often poorly preserved state of the archaeological hairs studied, which can be partial or display degradation causing morphological changes of the appearance of traits used for species identification. Moreover, the identification of archaeological hairs are carried out by comparison with atlases and reference collections consisting of modern animal hairs only. Such comparisons can cause problems as hairs for, at least, some species are known to have evolved during domestication and selective breeding. For instance prehistoric sheep had coarse kemp hairs covering a thin underwool which over time developed into the woolly coat of modern sheep breeds (Ryder 1988). Morphological species identification of skins by comparison to modern references is, therefore, also difficult as the appearance of the pelt has also changed in the meantime. The perception of what the skin of a specific animal species would have looked like should therefore also be reconsidered. For instance the prehistoric skins that today closest resemble cattle may actually be goat as seen in the results (see Table 2 in the included article).

Species identification of skins is, therefore, one of the cases in archaeology where traditional methods cannot always yield a conclusive answer and where biomolecular methods can be applied to give alternative and preferably more secure species identifications (Schlumbaum 2010, Vuissoz 2007, Hollemeyer 2008, 2012, Toniolo 2012, Kirby 2013).

The aim of the CTR-initiated project funded by KUF was, therefore, to investigate the reliablity of species identification by light microscopy, with DNA analysis and mass spectrometry (MS)-based peptide sequencing. A pilot study had already shown that, DNA analysis was not applicable to material from acidic bog contexts and that microscopy and MS-based peptide sequencing, in some cases, yielded conflicting species identifications on the same samples (Schmidt et al. 2011). As this pilot study demonstrated, species identification is not straightforward. The purpose of the article included here is, therefore, to follow up on this initial result and to investigate the problems of species identification further, and to develop a valid method for species identification of skin material from bog contexts. This was done by analysing more samples with light microscopy, MS-based peptide sequencing (performed by the present author) and adding a third method that combined scanning electron microscopy (SEM) and light microscopy to the material.

Material

The objects for the study, ten capes and a tunic, were selected among skin objects belonging to the National Museum of Denmark. The capes included in this study derive from the localities of Baunsø, Borremose I, Huldremose I, Karlby, Haraldskær, and Roum that are all situated in Jutland. Apart from the capes from these loacalities, a unique skin tunic was included. It was found in Møgelmose in Jutland. All included finds are dated to the centuries around the common Era (Table 8).

Locality	Parish (sogn)	District (herred)	County (amt)	Place no.	Museum no.	Calibrated 14C date BP (95,4% probability) and dating no.
Baunsø	Roum	Rinds	Viborg	130909**	NM D11103a	AD 20-220 Dating by cape b (Ua-33586)
Baunsø	Roum	Rinds	Viborg	130909**	NM D11103b	AD 20-220 (Ua-33586)
Baunsø	Roum	Rinds	Viborg	130909**	NM D11103c	AD 20-220 Dating by cape b (Ua-33586)
Borremose	Års	Års	Ålborg	120814-231	Borremose I, NM C26450	365-116 BC Dating by textile (AAR-11678)
Haraldskær	Skibet	Tørrild	Vejle	170909-37	NM 3705	508-211 BC (AAR-11659)
Huldremose	Nimtofte	Randers Nørre	Randers	140119-27	Huldremose I, NM C3471	350-41BC Dating by textile (AAR-11675)
Karlby	Karlby	Nørre	Randers	141009-26	D4854b	200 BC-AD 90, 170 BC-AD 140 Dating by textile (Ua-3998, Ua-3999)
Karlby	Karlby	Nørre	Randers	141009-26	D4854c	200 BC-AD 90, 170 BC-AD 140 Dating by textile (Ua-3998, Ua-3999)
Karlby	Karlby	Nørre	Randers	141009-26	D4854e	200 BC-AD 90, 170 BC-AD 140 Dating by textile (Ua-3998, Ua-3999)
Møgelmose	Jelling	Tørrild	Vejle	170904-170	16316	520-150 BC, AD 1-550* (OxA-1188, Ua-334)
Roum	Roum	Rinds	Viborg	130909**	C37412	50 BC-AD 80 (Ua-33584)

Tabel 8. The included skin objects and their datings. * The youngest dating is the most probable according to Ebbesen (Ebbesen 2009). ** Baunsø and Roum have no sb numbers.

From each skin element, three samples were taken[16] and sent to three different labs: 1) The Conservation Department of The National Museum of Denmark, Copenhagen, Denmark, performing macroscopic observations and light microscopy, 2) ArchaeoTex, Ennenda, Switzerland performing light microscopy and SEM and 3) Centre for GeoGenetics, University of Copenhagen, Denmark performing MS-based peptide sequencing.

MS-based peptide sequencing

The analysis of peptides by mass spectronomy was performed by the author at the Centre for GeoGenetics. The method takes advantage of peptides that display small structural sequence variations between different species (Brown and Brown 2011: 39). When such differencies are known, they enable the species from which a protein derives to be identified by comparison to online reference databases

[16] The sample from Haraldskær, NM 3705 was only analysed by two methods, as there were not enough hairs for both microscopic methods, whereas two elements from Huldremose I, NM C3471 were sampled.

of published protein and peptide sequences. Different methods for protein extraction and sequencing are in use (see method section). The method applied in this study is described in detail in Brandt et al. 2014, but the principles will be explained here briefly. Initially, proteins of a sample were extracted by mechanically and chemically breaking down the cell structure of the skin samples to allow its proteins to be solubilised. Through washes and centrifugion steps, unwanted parts of the cell and reagents were removed while the proteins were retained. By adding trypsin, an enzyme that digests proteins, protein residues were cut into peptides that were then immobilised in specific tips. The tips were sent to the Centre for Protein Research[17] where the peptides were sequenced using a mass spectrometer. The raw files produced were analysed at the Centre for GeoGenetics following the proceedure described in Brandt et al. 2014. The data included peptide sequences that were compared to available online databases enabling a 100% species match in most cases. Hereafter, the results from the MS-based peptide sequencing were compared to the results of the microscopical analysis.

The applied method has it strength in that it is also applicable to materials from highly degrading environments from which DNA has proven impossible to extract. This, for instance, makes the method valuable for archaeological bog contexts from which many of the ancient Danish textiles and skins derive. As the paper demonstrates, the method provides alternative information to that available from DNA.

MS-based peptide sequencing has yielded an alternative possibility for species identification of skin from bogs to microscopy, which, as we shall see, has provided significant results and perspectives for species identification in the future.

Conclusions and perspectives of the study

The results of the included article (Brandt et al. 2014) demonstrate that microscopy of ancient hair samples is challenging. The species identification results were fully compatible among all three methods for six of the twelve samples. In the remaining six cases, the microscopy-based methods consistently conflicted with each other, while the MS-based peptide sequencing agreed with one of the two microscopy-based methods in four of six cases. The conflicting results of the two microscopic methods are most likely caused by their different abilities to evaluate morphological hair traits used for the identifications. For instance the visual inspection of the skin applied at the Concervation Department of The National Museum of Denmark enables an initial categorisation of the animal by size and appearance of the entire skin. However, SEM, applied at ArchaeoTex, gives a much better resolution of

[17] The Novo Nordisk Foundation Center for Protein Research, Faculty of Health and Medical Sciences, University of Copenhagen.

the scales of the hairs, which is an important distiguishing trait. Due to these obvious challenges using microscopy, MS-based identifications have provided a valuable contribution to microscopic studies and highlighted some of the problem areas in the methods.

MS-based peptide sequencing showed itself to be a reliable method for species identification with several advantages. First of all, it is applicable to material from harsh environments, such as peat bogs, from which DNA is hard to retrieve. Second, the analysis yielded strong petide identifications that make the species identifications of mass spectronomy highly reliable. Third, mass spectrometry yielded several additional and novel results, including the finding of bovine fetal hemoglobin in one skin sample, indicating that the skin came from an animal slaughtered within the first month of its birth. This shows that MS-based peptide sequencing has the potential to yield information not obtainable by the analysis of ancient DNA. However, the method is still limited by the online availbility of proteomes and protein lists which is at the present limited to the most common domestic species. Moreover, the costs of mass spectronomy limits the number of samples that can be processed within most research budgets. From an archaeological point of view, it would, therefore, be optimal if microscopy was a reliable method for species identification, allowing larger sample sets to be analysed.

In the present study, only samples of closely related species, such as cattle and especially sheep and goats were tested. Although similar validation tests of other species have not been carried out, hairs of, for instance Arctic species (which are the topic of Chapter 3 and 4, and which includes species that are less closely related), are likely to be easier to distinguish by hair morphology.

At present, microscopy yields consistent results for sheep, whereas the identification of goats, horses and cattle seem more problematic. The included article (Brandt et al 2014) thus proves its worth in highlighting where the pitfalls in microscopic species identification occur, so that future studies can work on refining the identifications of these species.

The included study was setup with the scientific purpose of evaluating species identification methods. As mass spectrometry is an expensive and time-consuming method, this shows in the sampling strategy, where only one or two skin elements from each cape were species identified. Based on light microscopy and macroscopic observation, Mannering et al. 2011 state that the white skins of the outer cape from Huldremose are goatskin, whereas the brown are sheepskin. The analysis of a light and a dark skin from the other cape from the Huldremose find, the inner cape, which is included in Brandt et al. 2014, however, determined both as sheepskin. In this respect, it would be interesting to further species identify the skins of the outer cape, to revise if the skins reflect not only colour differences but also different species. Nevertheless, the result that both skin colours from the inner cape derive from sheep,

encourages a further analysis of the skins from the outer cape with MS-based peptide sequencing to ascertain whether the white skins are goatskin, and the brown sheepskin.

From an archaeological perspective, one could have wished to species identify all of the skin elements in one cape with MS-based peptide sequencing in order to ascertain if several species appeared in a single cape and if so, which elements they were chosen for. This would give a better indication of a potential relationship between the cape element and the properties of the selected skin, i.e. its size, thickness and flexibility (Harris 2006, 2012). This was performed by light microscopy and the analysis of the grain pattern (Schmidt and Mannering forthcoming), but not by MS-based peptide sequencing. The performed analysis showed that the capes were most often sewn together of skins from the same species.

Having dealt with methodological issues of species identification we will move onto the archaeological implications of the species identification. As mentioned in the introduction, the choice of animal skin has implications for its functionality, appearance, use, and perhaps significance, and provides information on animal exploitation. These aspects will be dealt with in the two following sections.

Implications of the use of skin from different animal species

Only 12 skin elements from 11 different garments were sampled for MS-based peptide sequencing. From this sample it is not possible to demonstrate any significant pattern in how different animal species were used for the production of skin garments. The analysis carried out by light microscopy and grain pattern analysis in the DTC research programme of all the available capes, however, confirms that domesticated animals clearly dominate over wild (Mannering and Gleba forthcoming). Apart from the 12 samples analysed, these identification have not been validated by MS-based microscopy. This analysis, however, validated that sheep, goats and cattle wer all utilised for skin clothing.

In the following, I discuss the functionality of the skins of these three domesticated animal species, as differences in skin properties could be an obvious reason to select one or the other species.

Structure and properties of animal skins

All species have different skin properties which influence, for instance the strength and flexibility of the skin (Haines 1991: 1). Mammalian skin has distinct layers: the thin outer layer, epidermis, the underlying dermis, and the hypodermis or flesh layer (Fig. 28). The dermis can be divided into the upper dermal papillary layer and the lower fibre network layer. The dermal papillary layer is a fibrous tissue which contains blood vessels, sweat glands and hair follicles (Reed 1966: 143). The fibre network layer constitutes the main part of the dermis, and is almost entirely made up by tissue fibres of which the

main component is collagen fibrils (Reed 1966: 144) (see later). Many of the properties of leather depend on exactly this layer (Reed 1972: 29). The grain pattern mentioned in Brandt et al. 2014 is the exposed surface of the dermal papillary layer after dehairing. Even after removing the hairs, the type and distribution of hairs can still be detected from the follicles. This pattern is characteristic for each animal species and can also be used for species identification (Reed 1972: 25).

The basic structure of all vertebrate skin consists of interwoven bundles of fibres of the protein collagen. Apart from this similiarity, animal species display distinctive skin structures and weave of fibre bundles.

Different species have varying skin thickness, size, angle of fibre bundles, and differing proportions of the skin thickness comprising the dermal papillary layer. This has considerable influence on properties, such as strength and flexibility of the skin (Haines 1991: 1). Skin properties may also vary within a single species depending on body part, breed, sex, age, nutrition,and geographical area (Reed 1972: 31-44).

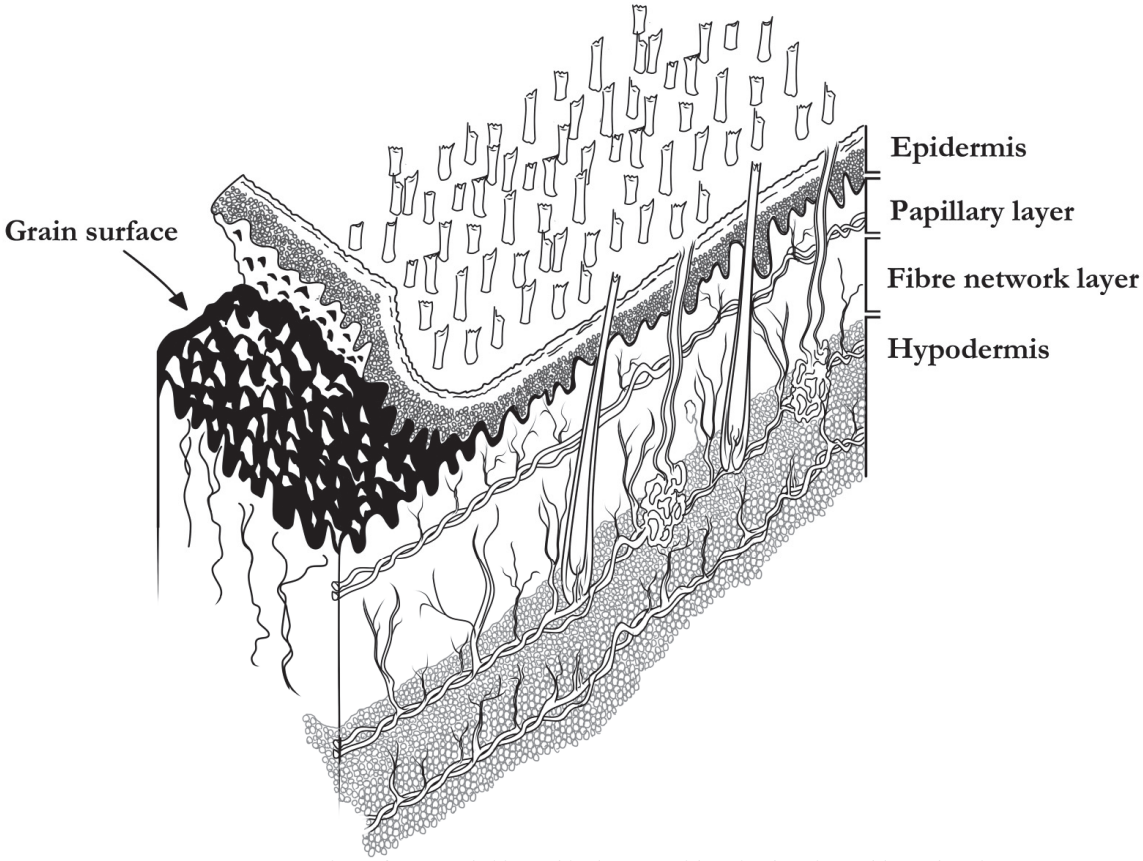

Figure 28. Cross section of mammal skin and its layers, epidermis, dermis, and hypodermis.
Drawing: Sidsel Frisch

Based on observations of modern animal skins, the skin of cattle, sheep and goats are all suitable for different purposes according to their properties (Haines 1991, Reed 1972). The modern skin of mature cattle is generally thick (4-6mm) and the dermal papillary layer constitutes only 1/6 of the dermis. The fibres of the fibre network layer are coarse and compact, which means that the hide is thick, firm and solid and prefered for items, such as soles and harnesses (Haines 2006: 13). This is also seen in Danish prehistoric shoes of the Bronze and Iron Ages, that are made of thick and strong cattle skin (Mannering et al. 2012: 101, Mannering and Gleba forthcoming). Compared to cattle, calfskin is thinner, from approximately 1-2,5 mm in the first year of its life (Haines 1991: 1-2). Apart from this, its properties resemble those of cattle, being strong due to the compactness of the fibre weave of the fibre network layer (Haines 2006: 14). Haines notes that calfskin is unsuitable for clothing due to its compactness and lack of softness (Haines 2006: 14). Therefore, it makes good sense that one of the cattle samples was determined as calf, as mature cattle skin would be much too thick for clothing.

Sheepskin varies according to the breed as does the coat of hairs and wool. The weave of fibres is generally more loose and the fibres finer than in goat or cattle skin, making sheep skin softer and drapable and well suited for clothing (Haines 2006: 15).

Goatskin displays fine fibres and compact weave of fibres, which makes it strong but less drapable than sheepskin and thus less suiable for clothing, but ideal for bookbinding,for instance (Reed 1972: 41).

The effects of skin processing for leather properties

Apart from the skins' inherent characteristics, the processing of skin also has an influence on the properties of skin and leather[18]. The purpose of processing skin is to avoid putrefaction and to strenghten the skins and isolate the collagen fibres to achieve a product suitable for its intended use (Reed 1972: 46, Larsen 1999: 35). Several tanning agents, including vegetable tannage, mineral tannage and fat tannage are known to have been used for millenia (Reed 1972: 86f).

Apart from these structural properties, the size of the animal naturally determines the size of skin available, while the appearance of the skin is determined by its hairs and/or the treatment processes it has gone through.

[18] According to Sykes, leather can be defined as tanned collagen (Sykes 1991).

Functionality of the capes

As described above, some skins seem better suitable for some purposes. An obvious question is, therefore, to what extent skins were chosen for specific properties, such as functionality and appearance. Were they produced and selected for specific purposes, or do the skin objects reflect the availability of skins from animals kept for other purposes?

The sewn together elements of the capes follow clearly standardised patterns which along with the elaborate seams, the cutting of the skins and the design of the capes demonstrates a refined craftsmanship (Mannering et al. 2011: 38, Schmidt and Mannering forthcoming). These features suggests a great intentionality in the production of the capes. Therefore, it seems likely that, also the choice and quality of skin were of great importance to obtain functional properties, as seen for the shoes, and were not merely picked for their availability. However, sheep, goa and cattle skin have all been used for the capes, with no clear pattern of being used for specific parts of the capes.

Appearance

Turning away from the functionality, the appearance of the cape also seems to have been important. Most capes would have looked like an entire skin from a distance, as the sewing would not have been visible. However, the brown outercoat of the Huldremose Woman displays a panel of three light skin elements at the top (Fig. 29, Mannering et al. 2012: 105-14, Mannering and Gleba forthcoming).

The light panel rested on the shoulder girdle, giving the effect of a collar (Hald 1980: 315). Apart from functional and stylistic considerations, it can be speculated that particular animal species had greater value and could be associated with better quality or a specific status. However, not sharing a cultural background with the wearers of the capes and understanding their social codes of dressing in a certain context, it is impossible to interpret how the use of skin was perceived, even though its signalling may have easily been noticed by the Danish Early Iron Age observers (Mannering et al. 2011:38).

Figure 29. The outer cape from Huldremose (C3472) with a light panel around the shoulders. Roberto Fortuna, the National Museum of Denmark.

Discussion of the long continuity of the skin capes

Although dated to a span of several centuries, the skin capes have suprisingly similar patterns. For the symmetrical capes, the design is similar over about 600 years, whereas inthe case of the asymmetrical capes, the pattern has remained constant for about 1100 years (Schmidt and Mannering forthcoming). Apart from the design, the sewing techniques and the fastening devices, too, do not change (Schmidt and Mannering forthcoming). The long continuity of the pattern of the capes is even more extraordinary as textiles display changes through the Pre Roman and Roman Iron Ages (Mannering et al. 2010, Mannering et al. 2012). The capes thus seem to follow a more conservative tradition than the textiles. This generates the question of whether the capes possessed functional properties so suitable for their pupose that they did not require major changes, but only minor stylistic ajustments. Presumably, the cutting of the skins aimed at utilising them in the most economic way, so that all pieces were used. Schmidt and Mannering suggest that the skin from the animals' back was used for the main part of the cape, as it was probably the most compact part of the skin (Larsen 1999, see Fig. 30). Skin from the limbs could be more suitablefor other parts of the capes that did not require such compact skin (Schmidt and Mannering forthcoming). Thus, the animal's entire skin was used and its properties were exploited in the best possible way. I believe that this optimal utilisation of the skins' properties and the design that utilises all parts of the skins are part of the explanation as to why the pattern of the cape did not change significantly over the centuries.

The apperance of the capes may also have been so familiar and essential in the Early Danish Iron Age society that the capes kept their overall design, although other garments and textiles changed their appearance. Clothes have been discussed extensively as a maker of identity. Apart from functional and stylistic considerations of the use of skin, studies of clothes and prehistoric costume have focused on the ability of costumes to signal different aspects of identity such as sex, age, ethnicity, religion and status (Harlow 2012). Such signals may be clear to people sharing the same cultural background and understanding of such dress codes, whereas finer nuances may be harder to pick up even for people sharing the same cultural background. Understanding the meaning of dress may, therefore, be even harder for people not sharing cultural or temporal backgrounds (Harlow 2012). As the communication of identity via dress is an area studied extensively and requiring a large set of methodologicl and theoretical tools to understand sources of dress and their interpretation, it will be noted here only briefly that, the choices of using specific skins may be a way to display specific aspects of identity, and that the long continuity of the capes could be explained by their easily read communication of the wearers' identity.

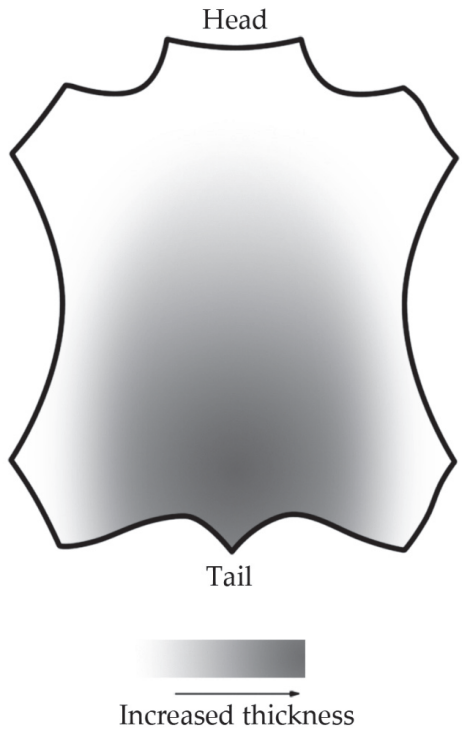

Figure 30. The thickness of animal skin, after Larsen 1999.

The capes discussed are only found on bog bodies which have previously been interpreted as those who in one way or the other stood out from the rest of society (Ravn 2010). The bare fact that the bog people did not receive the same funeral as the majority of the population makes them stand out. In the Pre Roman Iron Age cremation graves dominate completely. In the Early Roman Iron Age, cremation graves still continue to predominate, while inhumation graves become more common and dominate in the Late Roman Iron Age (Hedeager 1992: 99-100). As only very few remains of textiles are preserved from the Pre Roman Iron Age, it is difficult to compare the clothes of people buried in bogs with people who received the more common form of burial. However, judging from the few textiles found in graves of the Pre Roman Iron Age compared to textiles found in bogs there is no discernible difference in the clothing of these two groups of people that could suggest that bog people stood apart (Bender Jørgensen 1986: 26-28).

The opinion that bog bodies made up a specific group in the Early Danish Iron Age particularly derives from interpretations of a paragraph from *De origine et situ Germanorum* by Tacitus in which he describes the crimes for which people could be punished by being drowned in peat bogs (Tacitus, *De origine et situ Germanorum* 1.12.1 (Bruun and Lund 1974a: 47). The different interpretations of the bog bodies span from executed individuals to sacrifices (Ravn 2010). One of the crimes described by Tacitus, that would lead to being drowned in bogs is described as "corpore infame" (Tacitus, *De origine et situ Germanorum* 1.12.1). The interpretation of this concept has been discussed extensively, but the translation: "…those who have defiled their bodies…"[19] is today widely recognised as referring to homosexuality (Bruun and Lund 1974b: 22, Ravn 2010: 109). There has, therefore, been great focus on homosexuality in relation to the bog bodies.

The interpretation of bog bodies as outcasts has been supported by many of the bodies having been found naked, with traces of severe violence, and buried in a manner different tothe predominant manner of the times (see above). New pathological studies have, however, demonstrated that most of the violent traumas are caused by the peat diggers who discovered the bodies (Asingh and Lynnerup 2007: 286-287), or the pressure and movement of the peat working on the fragile bones and causing them to break (Fisher 2007: 116). Yet, some of the traumas cannot be explained away as in the case of, for instance the rope around the neck of the Tollund Man and the skull trauma of the Borremose Man.

New studies lead to a possibility that naked bog bodies could have been dressed as well. Close studies of the Huldremose Woman have revealed plant fibres on her skin, demonstrating that the bodies could be dressed in plant fibre materials, which are more prone to degrade in the peat bogs (Frei et al 2009b: 1966). This means that, for instance the Tollund Man, who was found naked apart from a leather belt and hat, could have worn a plant fibre garment which would explain the belt.

Moreover, Ravn points to artefacts associated with the bodies that may be interpreted as grave goods (Ravn 2010: 111). Ravn thus concludes that the deposition of bog bodies cannot be referred to a single explanation but may rather be a combination of several, such as sacrifices, executions and simple natural causes of death with subsequent burial in a peat bog (Ravn 2010: 113).

Tacitus mentions in *De origine et situ Germanorum* that, the common clothing of Germanic people was a cape. However, he does not clearly describe their material (Tacitus, *De origine et situ Germanorum* 17.1 (Bruun and Lund 1974: 9). He does, however, state that Germanic people wear skins of wild animals (Tacitus, *De origine et situ Germanorum* 12.2,Bruun and Lund 1974: 53). *De origine et situ Germanorum* was

[19] (author's own translation)…kujoner, krystere og sådanne, der har vanæret deres krop, drukner de i sumpede moser og lægger et fletværk over dem

published in AD 98 which falls within the time span to which the Danish skin capes have been dated. Tacitus was never in Germania himself but has probably used written sources available at the time of writing and communicated with first-hand sources for his description of Germania in the first century AD (Bruun and Lund 1974a: 24-27). In the instances where statements of Tacitus can be tested, he is believed to provide a fairly good depiction of Germania in the first century AD (Bruun og Lund 1974a: 27). However, the tribes he describe are North Germanic and not Scandinavian and the society he describes may, therefore, display similiarities to the Scandinavian, but is not necessarily the same. Thus, there is good reason to be cautious when using Tacitus as a source for Danish Iron Age conditions. *De origine et situ Germanorum* is, however, one of the only available written sources to first-century Germania and therefore provides a much needed source to compare archaeological research to as also pictoral evidence of humans from Scandinavia is scarce in this time period (Hald 1950: 326, Hald 1980: 320).
Another source, providing information on the Germanic world which was contemporaneous with the Early Danish Iron Age is Caius Julius Caesars work *Commentarii de Bello Gallico*. Books I-VII of this work were written in the winter of 52-51 BC and comprise observations made in Gaul during the Gallic Wars (58-52 BC) (Kock 1922: 23). As with *De origine et situ Germanorum,* the work is often included as it provides much needed comparative statements. Like Tacitus, Julius Caesar also mentions that Germanic people dressed in skins and wore short garments leaving a great part of the body naked (Caesar, *Commentarii de Bello Gallico* 6.21 (Kock 1922: 212)).
Judging from the finds of clothing, the cape was common in Denmark in the Early Iron Age as Tacitus and Caesar mention was also the case for the Germanic people. Tacitus's statement of them wearing skins of wild animals is, however, not something we can confirm based on the Danish skin capes, as the analysis indicates the great majority of the skins actually derive from domesticated animals.

Whether the capes were not actual clothing but specifically given to the deceased for the burial have also been discussed. According to find records, several capes were not placed on the bog bodies as they would have been worn (Hald 1980: 313). The cape from Daugbjerg was placed with the collar towards the feet and several capes were more likely used for wrapping the bodies or as covers than dressing them (Hald 1980: 313). Moreover, several capes were found in connection to only one body, as is the case for Baunsø and Karlby where three and four capes respectively were found in association with one body. However, many of the capes show evidence of repair with new patches, demonstrating wear and tear. A cape from Karlby (cape D4854b) had been enlarged by adding a lapel and collar indicating that the capes could follow a person during his growth or be passed on. Such information shows that the capes were not merely shrouds.

The amount of skin used for one cape was considerable. The number of skins used in one cape represents several animals and the work producing it must have been time consuming. The innercape of the Huldremose Woman was repaired with 22 patches, several of these overlapping which indicates that the cape was repaired continously (Mannering et al. 2010). Therefore, the capes must have represented a great value to its owner. The capes, however, do not necessarily express status, as the wear indicates that they were used over an extended period of time.

From the availble material of clothing from graves and depictions of clothes, it is impossible to determine whether the capes belonged to a certain part of the population (Hald 1980). Judging from the repair and changes on several capes, the capes were not merely funerary clothing but also worn during life. Their properties would also have made them highly suited for the wet and cold weather, shielding the wearer from rain with the hairs worn outside and pointing downwards, and worn on top of each other in cold weather. No other garment type with these qualities is known from the find records. Square wool textiles could have been worn as shawls, but would not have had the protecting effect against rain as these absorb water instead of repelling it.

The manufacture and pattern of the capes points to a well developed craftmanship and the choice of design is very likely to be connected to the way the skins could be utilised most efficiently, and at the same time, suiting the specific requirements of parts of the capes. The knowledge of their apperance and production must have been passed down through generations. The considerations of efficient utilisation may be part of their long contituity along with their excellent functionality. Apart from this, it cannot be ruled out that their design held a certain significance for their contemporary cultural context, that we are not able to ascertain today.

Socio-economic perspectives of animal skin exploitation

The identification of animal species used for skin garments provides information about the range of animals that was part of the prehistoric economy. Interestingly, species identification of skin objects can reveal whether both wild and domesticated animals were used for skin clothes and supplement the knowledge on what products were exploited from which animals. All the skins analysed in the included article derive from domesticated animals. The majority (over 90%) of the skins from the Danish bogs were previously species identified by microscopy to domesticated animals. However, several wild animals species were also identified. Beaver (Castor) and wolf or dog (*Canis*) were identified in cape c from Karlby (Hald 1950: 42), deer (*Cervus*) was identified in the cape from Refstrup Hovedgaard

(Ebbesen 2009: 42), and the genus (*Martes*[20]) was previously identified in the tunic from Møgelmose (Ebbesen 2009: 41). The dominance of domestic animals amongst the analysed skins corresponds with the pattern seen in examined bone assemblages from contemporary Pre Roman and Early Roman Iron Age settlement sites. These demonstrate that, wild animals only seem to play a small role as game in the Iron Age and that the economy relied primarily on the domestic animals (Kveiborg 2008: 61).

Kill-off patterns (see chapter 1), have been a primary source for theories on animal exploitation. The main objectives for animal husbandry have often been described as meat and milk, and for sheep wool, and the age of slaughter has been explained as being indicative of a focus on these products. This is interesting, as these are the products perhaps most appreciated in today's society where raw materials for textiles and garments not only derive from many animal resources but also from artificial fibres. The modern focus on prehistoric animal products may thus be biased by the values of modern researchers. Prehistoric societies must have had a large demand for raw materials for textile and garment production and skin must have been a precious material and perhaps a more appreciated animal product than previously believed.

However, animals that died during the first month of their lives are often interpreted as slaughtered for their delicious meat. This theory is strengthened by the awareness of offerings of young animals in graves of the Roman Iron Age (Gotfredsen in press). However, the finding of foetal heamoglobin in the skin from Møgelmose now questions if this was the only reason for the slaughtering of young animals. Skin from young animals may have been an equally important product as meat. Today, skins from young animals are often preferred for their softness and the skin from Møgelmose proves that young animals were used for skin clothing. That calfskin is seen in a garment contrary to mature cattle skin accords well with the skin properties of these ages, as cattle skin would have been too thick. Other skins determined as cattle could very well be calf, although foetal heamoglobin was not recovered from these. However, it cannot be ruled out that animals were slaughtered at a young age as a resource for both meat and skin of high quality.

That not only young animals were utilised is apparent from the goatskin from Baunsø a, which would not have had the large size it does, if it had only derived from a young animal.

Although the results of the proteomic analysis are too few to yield any conclusive answers, they give rise to a more refined discussion of the exploitation of animal resources and the values of different animal products. Essentially, these can now also be investigated through another source: skin garments. Whereas the analysis of bone assemblages (see chapter 1) have most often been used to evaluate what a

[20] From a skin element from the cape from Møgelmose.

production was intended for, typically meat, milk or wool, in this case, another product, skin, which is most often not considered, is now available as direct evidence for the species and, in some cases, also the age of the animals that were used in skin production.

Conclusions

Mass Spectrometry-based petide sequencing was demonstrated to be a secure method for species identification. However, the method is not applicable to a large sample set. Moreover, the archaeological world is often limited by the financial ressources set aside for scientific analysis. As the analysis demonstrated inconsistencies between the species identifications, I do not believe that the application of MS-based peptide sequencing was excessive in this case. Indeed, I believe that it is a strong and indispensable tool in cases of doubt and between closely related species. Furthermore, an option already mentioned is to make a reference collection of archaeological material, which will hopefully yield better comparisons. Such a reference collectin is already being built by Antoinette Rast-Eicher, who performed one of the microscopic analysis. However, this has not yet been done with Mass Spectrometry-based peptide sequencing. The results of this study suggest precisely such a course of acting. MS-based peptide sequencing could then be used for samples of particular difficulty or with significant relevance.

Article

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Species Identification of Archaeological Skin Objects from Danish Bogs: Comparison between Mass Spectrometry-Based Peptide Sequencing and Microscopy-Based Methods

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Abstract

Denmark has an extraordinarily large and well-preserved collection of archaeological skin garments found in peat bogs, dated to approximately 920 BC – AD 775. These objects provide not only the possibility to study prehistoric skin costume and technologies, but also to investigate the animal species used for the production of skin garments. Until recently, species identification of archaeological skin was primarily performed by light and scanning electron microscopy or the analysis of ancient DNA. However, the efficacy of these methods can be limited due to the harsh, mostly acidic environment of peat bogs leading to morphological and molecular degradation within the samples. We compared species assignment results of twelve archaeological skin samples from Danish bogs using Mass Spectrometry (MS)-based peptide sequencing, against results obtained using light and scanning electron microscopy. While it was difficult to obtain reliable results using microscopy, MS enabled the identification of several species-diagnostic peptides, mostly from collagen and keratins, allowing confident species discrimination even among taxonomically close organisms, such as sheep and goat. Unlike previous MS-based methods, mostly relying on peptide fingerprinting, the shotgun sequencing approach we describe aims to identify the complete extracted ancient proteome, without preselected specific targets. As an example, we report the identification, in one of the samples, of two peptides uniquely assigned to bovine foetal haemoglobin, indicating the production of skin from a calf slaughtered within the first months of its life. We conclude that MS-based peptide sequencing is a reliable method for species identification of samples from bogs. The mass spectrometry proteomics data were deposited in the ProteomeXchange Consortium with the dataset identifier PXD001029.

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. The mass spectrometry proteomics data were deposited in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the data set identifier PXD001029.

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Introduction

BACKGROUND

Skin and leather artefacts are rarely found in archaeological contexts, as biogenic and non-biogenic factors rapidly cause their complete decomposition [1]. Nevertheless, archaeological objects that derive from animal soft tissues, such as skin and leather, may survive in environments with exceptional conditions, such as anoxia, waterlogging, low temperature, high salt concentration, or extreme dryness [2,3]. One favourable environment in this regard is the raised bogs of North Western Europe, as their acidic and anaerobic soil with low average temperature and content of sphagnan inhibit microorganism proliferation and promote skin, hair, and other soft tissue preservation by natural tanning processes [4]. Therefore, a significant number of deposited ancient textiles and skin garments had been preserved in raised bogs and unearthed during peat cutting [5–7].

Danish peat bogs, in particular, have yielded one of the world's finest collections of prehistoric textiles and skins, including more than 68 well-preserved prehistoric skin objects [8,9] dating from 920 BC to AD 775 approximately, i.e. the Danish Late Bronze and Iron Ages [10,11]. The skin object collection predominantly consists of capes and shoes, and while some retain nearly full hair content, others lack parts, or all of the original hair. The 24 skin capes, found either singly or associated with male or female bog bodies, are considered to represent unisex clothing [5]. The capes were sewn together of 4–7 large polygons or rectangular elements,

each representing an entire animal skin, and several smaller pieces of skin. The majority of the capes are symmetrically designed, whereas a minor part displays an asymmetric design (Fig. S1 in File S1). The largest skin elements measure up to approximately 90 cm in height, but on average they measure between approximately 30–50 cm in height, and 25–40 cm in width. An essential feature noted on some of the skin elements is the dorsal line of hair often placed in the centre of the elements, indicating that skins were cut symmetrically along the spine of the animal, which today, too, is the customary manner of cutting an animal skin.

These exceptional finds provide a unique opportunity to not only investigate prehistoric skin costume technologies, but also understand which animal species were used in the process. This is crucial as species-specific morphological characteristics of skins, such as size, thickness, flexibility and function determine costume properties and the number of elements required to produce a skin object [12–14]. The types of skin utilized also define the pertinent manufacturing techniques and possible product types. Moreover, species identification of archaeological skins can enhance our understanding of prehistoric animal husbandry. This includes the exploitation and preferences of animal products as meat, milk, wool and skins, and the management strategies of flocks required to produce these products.

PREVIOUS METHODS FOR SPECIES IDENTIFICATION OF ARCHAEOLOGICAL SKIN OBJECTS

Attempts to identify the species origin of archaeological skin objects have been carried out since the 19th century [15]. Until recently, skins with a preserved pelage were primarily subjected to identification via either macroscopic inspection, or by using light and electronic microscopy to investigate the hair morphology [9,16–20]. This method is also extensively applied in forensic science [21,22]. The distribution of primary and secondary hairs is characteristic for each animal species, and the position and size of the various hairs produce a species-specific surface pattern of the grain or the dermal papillary layer, that varies over the body. The recognition of this so-called "grain pattern" is a further feature that can be used for the identification of animal species [14]. Grain pattern is primarily used on de-haired skin or fur skin with lost pelage. The recognition of the cross-section of the dermal layer by means of light microscopy can also be employed as a tool for the identification of animal species [14,23], however as skin sampling was restricted, this analysis was not included in this work.

Species identification is also commonly performed utilizing hair through the evaluation of 'diagnostic' morphological traits, including: hair diameter, length of the fibre, shape and distance of the cuticles scales, appearance and dimension of the medulla and cortex [24,25], and cross-sectional shape. These traits are evaluated and identified by comparison to atlases and reference collections [16,17,25–29]. Thus far, the majority of the skins of the Danish capes have been identified by microscopy as domesticated animals, such as sheep, goat and cattle. Otter (*Lutra lutra*) and wolf (*Canis lupus*) skins were, however, also identified in one cape, and deerskin (*Cervus*) in another [8]. Despite being widely applied, the reliability of species identification based on the light and electron microscopic observation of skin and hair morphology is subject to intense debate [16,17,24,25,30,31]. A primary matter of concern is that the reproducibility of the method requires extended knowledge and experience. Furthermore, hair morphology can diverge within the same species, between different parts of the animal's surface, or according to age, sex, seasonality, nutrition and health. These challenges are further complicated in archaeological contexts. First, fiber atlases are based on modern species and at present there is no fiber atlas available that includes archaeological material. This is problematic, as domestication and selective breeding of animals have altered hair morphology, which is reflected in the appearances of the scale structure and medulla [17,32,33]. Secondly, archaeological hairs are often poorly preserved [34] and the degradation of prehistoric hairs can transform the appearance of the scales and medulla, which complicates the identifications [35–37]. Thirdly, environmental conditions can lead to the preservation of only partial fibres. These can yield misleading identifications, as scales and types of primary follicles differ, to some extent, between areas of the hair. Overall, it is evident that species identification based on the microscopic analysis of ancient hairs is not straightforward, thus rendering it desirable to develop alternative, ideally more reliable, approaches for the species identification of skins.

In recent decades, new methods based on the analyses of ancient biomolecules have been applied for the species identification of hide and leather. An ancient DNA-based approach was successfully applied to ancient parchment, bookbinding and clothing of hide and leather [38,39]. The success of DNA-based approaches, however, depends on DNA preservation, which is conditioned by the diagenetic conditions that the sample experienced during archaeological deposition. The acidity and generally high amounts of molecules identified as PCR inhibitors in peat bogs affect aDNA preservation and strongly hampers its potential for amplification by PCR [8,40,41]. This is equally the case for skins and textiles that have been subject to tanning or mordanting processes [39,42].

More recently, an alternative molecular approach for species identification, adopting mass spectrometry (MS) to analyse collagen and keratin residues extracted from small archaeological bone fragments, as well as skin and fur, was presented [43–51]. Collagen preservation levels in ancient skin objects, associated with highly hierarchical structural constraints and macroscopic protein quantities, suggest that, MS-based ancient peptide sequencing is applicable to samples from bogs, despite their exposure to harsh diagenetic conditions. Recently, methodological improvements and protocol optimisation, taking ancient protein characteristics into account, have enabled the identification of considerably more proteins than achievable hitherto [52–54]. Moreover, protein analysis holds the advantage of not being based on enzymatic amplification and consequently not being affected by conventional PCR inhibitors, overcoming the limits of aDNA analysis from ancient recalcitrant contexts [8].

We explored the potential of MS-based high throughput ancient peptide sequencing as a reliable approach for the species identification of archaeological skin objects from peat bogs. Unlike previous methods based on mass fingerprinting of peptides from selected collagen and keratin molecules, the shotgun sequencing approach aims to identify the total extracted ancient proteome, with no specific target selected in advance. In this study, we subjected samples from eleven archaeological skin objects to species identification employing three different approaches. Two of these rely on microscopy: the first combines macroscopic observation (MO) of the skin and inspection of the associated hairs by light microscopy (LM), while the second adopts light and scanning electron microscopy (SEM)-based observation of the hair morphology. The third approach is based on ancient peptide sequencing by MS. The conclusions reached by the three methods are compared, and the advantages and limitations of the various approaches discussed.

Materials

Twelve samples from eleven skin garments (ten capes and a tunic) from seven peat bog localities in Denmark were selected for this study (Fig. 1, Table 1, Fig. S1 in File S1). All samples derive from the collection of skin objects at the National Museum of Denmark. The dataset for each garment (except for the Huldremose I find, for which two samples were collected from two different skin elements) consisted of three samples extracted from the same skin element, as these sewn together skin elements may derive from different species. A skin sample, measuring approximately 2×2 mm, was cut off for MS-based peptide sequencing, together with a few hairs for microscopy analyses (Fig. S1 in File S1). To validate the MS approach, three modern reference samples were also analysed (Table 1), representing the three common domesticated species that the archaeological samples most likely derived from (cow, goat, sheep). The references were sampled from two historic skin samples from the Natural History Museum of Denmark, known to derive from domestic sheep and goats, and from a cattle skin provided by a local slaughterhouse.

Ethics Statement

The archaeological samples (1–12, Table 1) were provided by the National Museum of Denmark, Frederiksholms Kanal 12, DK-1220 Copenhagen K. The historical samples (CN3213 and CN3196) were obtained from the Natural History Museum of Denmark, Zoological Museum, Universitetsparken 15, DK-2100 Copenhagen Ø. All necessary permits were acquired for the described study, which complied with all relevant regulations. The modern cattle sample was obtained with the kind permission of Lennart Engberg Carlsen from the slaughterhouse Anubis, Department of Basic Animal and Veterinary Sciences, Grønnegårdsvej 7, DK-1870 Frederiksberg.

Data deposition note

The mass spectrometry proteomics data were deposited in the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository [55] with the data set identifier PXD001029.

Methods

Three different methods were applied to the same skin elements. Microscopy-based method 1 "MO+LM" was performed by Anne Lisbeth Schmidt, at the National Museum of Denmark's Conservation Department, microscopy-based method 2 "LM+SEM" by Antoinette Rast-Eicher, at ArchaeoTex, Switzerland, and MS-based ancient peptide sequencing method 3 "MS" was performed at the Centre for GeoGenetics by Luise Ørsted Brandt and Enrico Cappellini.

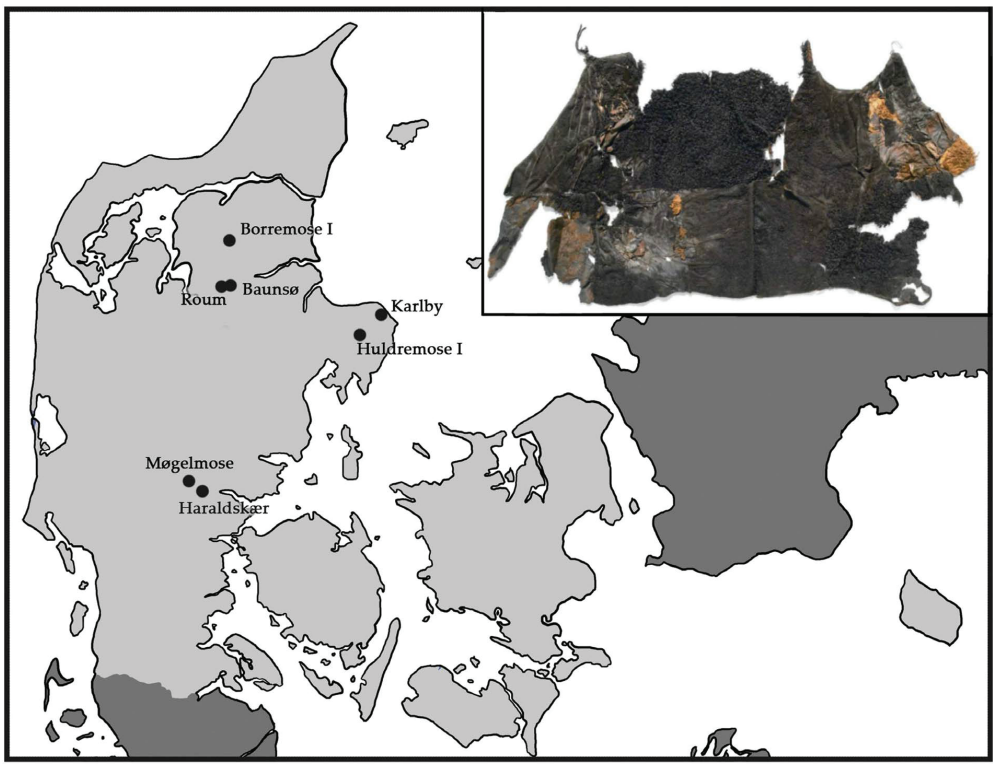

Figure 1. Locations where the investigated archaeological skin objects were found. Appearance of the skin cape from Huldremose I (inset). Photo by Roberto Fortuna, the National Museum of Denmark.
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Table 1. Archaeological skin objects from Danish peat bogs, and modern control samples investigated.

Sample no.	Locality	Museum no.	Calibrated 14C date BP	Dating
			(95, 4% probability)	
1	Baunsø*	NM D11103a	AD 20–220	
			Dating by cape b (Ua-33586)	
2	Baunsø	NM D11103b	AD 20–220	Ua-33586
3	Baunsø	NM D11103c	AD 20–220	-
			Dating by cape b (Ua-33586)	
4	Borremose I*	NM C26450	365–116 BC	
			Dating by textile (AAR-11678)	
5	Huldremose dark*	NM C3471	350–41BC	-
			Dating by textile (AAR-11675)	
6	Huldremose light*	NM C3471	350–41BC	-
			Dating by textile (AAR-11675)	
7	Karlby	NM D4854b	200 BC–AD 90, 170 BC–AD 140	-
			Dating by textile (Ua-3998, Ua-3999)	
8	Karlby	NM D4854c	200 BC–AD 90, 170 BC–AD 140	-
			Dating by textile (Ua-3998, Ua-3999)	
9	Karlby	NM D4854e	200 BC–AD 90, 170 BC–AD 140	-
			Dating by textile (Ua-3998, Ua-3999)	
10	Møgelmose*	NM 16316	520–150 BC, AD 1–550**	OxA-1188, Ua-334
11	Roum	NM C37412	50 BC–AD 80	Ua-33584
12	Haraldskær*	NM 3705	508–211 BC	AAR-11659
	Domestic sheep	CN3213	Dating not performed	Sampled in 1959
	(*Ovis aries*)			
	Domestic goat	CN3196	Dating not performed	Sampled in 1959
	(*Capra hircus*)			
	Domestic cattle	-	Dating not performed	Sampled in 2012
	(*Bos taurus*)			

The archaeological skin objects date to the Pre-Roman Iron Age: 500–1 BC, Early Roman Iron Age: AD 1–200, and Late Roman Iron Age: AD 200–400 [10].
* Samples prepared following procedure A during MS-peptide sequencing analysis.
** The youngest dating is the most probable according to Ebbesen [11].
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Species identification by microscopy and macroscopic observation

Two microscopy methods were applied for the purpose of traditional species identification. Both species identification methods used light microscopy: the first method 1, "MO+LM", combined light microscopy with macroscopic observation of the skins elements, whereas the second method, "LM+SEM", combined light microscopy with SEM.

Transmitted light microscopy focused on the observation of primary and secondary hair [16,29,56,57]. Cross-sectional photos were taken with an Axio Scan.Z1 Slide Scanner from Carl Zeiss Microscopy. Species identification was based on scale pattern and absence/presence and shape of medulla, according to the terminology of Wildman [25], and the shape of cross-sectioned hair, according to Teerink [16]. As reference, a range of fibre atlases was used [16,25–29], in combination with modern mammalian hair samples, which were kindly lent by the Natural History Museum of Denmark. In the present study the grain pattern was investigated for the sole de-haired sample 12 (Haraldskær NM3705).

Macroscopic observations of skin size, thickness and flexibility, as well as the general appearance of the hair in the pelage, were also applied in "MO+LM" to support the species identifications [8]. The appearance comprises hair length, shape, the presence or absence of hair curls, primary and secondary hair and dorsal hair stripes.

SEM analysis was restricted to hair samples [58,59], through comparison against several atlases and a private collection of reference samples [17,30,60] (Fig. S2 in File S1), in combination with an initial identification by light microscopy. The primary criteria for hair micromorphology-based identification of the commonest domesticated species (sheep, goat, cattle and horse) following Meyer et al. [17] are listed in Table S1 in File S1.

Species identification by MS-based ancient peptide sequencing

The third method used mass spectrometry to sequence ancient protein residues. The samples were analysed in two distinct batches adopting different sample preparation approaches. Conditions adopted for liquid chromatography-electrospray ionisation (LC-ESI) and high-resolution tandem mass spectrometry (MS/MS) are described in details as Supplementary Information (see Text S1 in File S1) and referred to as procedure 'A' and 'B'. Samples marked with an '*' symbol in Table 1 were prepared

following procedure 'A', while all the other samples were prepared following procedure 'B'.

Results

Species identification results were fully compatible in all three methods for six of the twelve samples (Table 2). In the remaining six cases, while the microscopy-based methods consistently disagree with each other, the MS-based peptide sequencing agrees with one of the two microscopy-based methods in four out of six cases. The three methods generally agree on the identifications of sheep (sample 3–7, 11) except for sample 9, in this case "LM+SEM" suggests a discordant identification. For the identification of other species, consensus seems harder to reach. In one case (sample 1), the three methods reached three different conclusions. In the case of sample 12, "MO+LM" and "MS" reached different results, whereas "LM+SEM" was not applicable as hair for only one microscopic analysis was available. In cases where "LM+SEM" and "MS" identified cattle, "MO+LM" identified goatskin (sample 2 and 10). In two cases "LM+SEM" and "MS" disagree between horse and goat identifications (sample 1 and 8).

The sample preparation procedure used for MS-based ancient peptide sequencing yielded protein recoveries estimated in the range between 1.32 and 20.13 mg of protein/g of extracted skin (Table S2 in File S1). While yields for proteins extracted from ancient skins have not been reported earlier, these values appear to be similar or superior to the approximately 5 mg protein/g bone obtained from ancient bone [52]. Skin samples from the same localities present similar values, suggesting that the protein yield could be related to archaeological site-specific preservation conditions. Statistics, reporting numbers of identified proteins and peptides for each sample, as well as the relative supporting tandem MS spectra, indicate that sample preparations based on procedure "A" enabled the recovery of richer datasets (Table S2 in File S1). Most of the proteins identified are collagens and keratins, in agreement with the nature of the samples analysed. However, the adopted approach also allowed the identification of proteins and peptides not previously reported in ancient skin samples [43], such as, leucine-rich-containing protein, serum albumin, selenium-binding protein and haemoglobin foetal subunit beta (Tables S3 in File S1).

The search strategy adopted enabled the determination of a set of species-specific peptides (Tables S3, S4 and S5 in File S1), within publicly available protein databases. Based on spectra matched against the complete bovine reference protein list and extended lists of sheep and goat proteins available in NCBI RefSeq (http://www.ncbi.nlm.nih.gov/refseq), it was possible to identify at least one species-diagnostic peptide for all samples except two: 9 and 11. Peptides were considered diagnostic when, after BLAST search [61] against the entire nrNCBI protein database, they were assigned to a single species, or to a limited number of species among which only one can be considered plausible, based on the nature of the samples, such as the size of the skin element, or their geographic origin. For example, peptides equally present in cattle (*Bos taurus*), water buffalo (*Bubalus bubalis*) and yak (*Bos mutus*) were considered diagnostic for cattle. For samples lacking at least one species-diagnostic peptide, i.e. sample 9 and 11, species identification was attempted based on a set of peptides [43,62], only compatible with one species (Fig. 2 and Table S5 and S6 in File S1).

Two skin samples were identified as bovine (sample 2 and 10), six as sheep (*Ovis aries*, sample 3–7 and 9), and three as goat (*Capra hircus,* sample 1, 8, 12), while for one sample (sample 11), identified as ovine, it was not possible to detect any marker to discriminate between sheep and goatskin (Table 2). The preparation of modern comparable material from known species with the same procedure, enabled the recovery of a higher number of diagnostic peptides (Tables S4 and S5 in File S1) for each sample. This is in full agreement with the recent origin of the material and its storage in favourable conditions. Only a limited number of the species-specific peptides identified (Fig. 2 and Table S5 in File S1), were previously reported in literature describing ancient samples [43,46–49,63].

The MS-based approach recovers additional information of particular interest for archaeological reconstruction and the understanding of the exploitation of natural resources in antiquity. An example is the secure identification of peptides uniquely assigned to bovine haemoglobin foetal subunit beta (UniProt accession number: P02081) in sample 10 (Fig. 3 and Table S3 in File S1). This protein is expressed in the foetus during the final months of pre-birth development and in those immediately after. At birth it represents approximately 40 to 100% of the total haemoglobin in a calf, and its concentration then diminishes rapidly until completely replaced by adult haemoglobins on average approximately two to three months after birth [64]. The identification of a protein expressed in such a defined time frame during pre- and immediately post-natal calf development allows a precise pinpointing of the time at which the animal was slaughtered for garment production. Although bovine haemoglobin is usually listed as a common proteomics contaminant, the absence of haemoglobin foetal subunit beta-specific peptides (reported in Fig. 3 and Table S3 in File S1) in all the other samples analysed in the same batch and in negative controls strongly suggests that these peptides were genuinely recovered from the archaeological sample and not indicators of a contamination. At present and to the best of our knowledge, there is no other approach that can provide this type of information for archaeological skin samples.

Discussion

The lack of consensus among the results of the microscopy-based methods, for half of the analysed samples, illustrates that their use as a tool in species identification is not straightforward. Most likely, the challenges hampering the macroscopic and microscopic identification of archaeological skins and hair constitute part of the explanation for these discrepancies. However, the two microscopic methods applied hold different advantages for species identification. Light microscopy provides information on the colour or pigmentation of the hair and the structure of the medulla, while SEM allows enhanced observation of the scale patterns due to high magnification and a 3D view. The macroscopic observation of the size and thickness of skin elements immediately excludes several species from further consideration. For instance, sample 1 was previously identified as deer [65] and during this study it was assigned to three different species: cattle, horse, and goat. The species identification of this skin therefore seems particularly difficult. The size of the skin element, 70 cm in length from neck to tail, is compatible with cattle and traditional Danish goat breeds [66], while its thickness and hair length leads to its identification as cattle skin. However, the absence of an accurately identified archaeological skin reference material cannot completely exclude ambiguous conclusions based on the observation of these traits.

The result of "LM+SEM" suggested that the skin in question was horse skin (Fig. S3). However, distinguishing between horse and goatskin with light microscopy and SEM is difficult as illustrated in Table S1 and Fig. S4 in File S1. MS-based

Table 2. Species identification of the archaeological skin samples, based on the three methods applied.

Sample no.	Find	MO+LM	LM+SEM	MS	MO+LM vs LM+SEM	MO+LM vs MS	LM+SEM vs MS
1	Baunsø, NM D11103a	Cattle	Horse	Goat	≠	≠	≠
2	Baunsø, NM D11103b	Goat	Cattle	Cattle	≠	≠	=
3	Baunsø, NM D11103c	Sheep	Sheep	Sheep	=	=	=
4	Borremose I, NM C26450	Sheep	Sheep	Sheep	=	=	=
5	Huldremose I dark, NM C3471	Sheep	Sheep	Sheep	=	=	=
6	Huldremose I light, NM C3471	Sheep	Sheep	Sheep	=	=	=
7	Karlby, NM D4854b	Sheep	Sheep	Sheep	=	=	=
8	Karlby, NM D4854c	Goat	Horse	Goat	≠	=	≠
9	Karlby, NM D4854e	Sheep	Cattle	Sheep	≠	=	≠
10	Møgelmose, NM 16316	Goat	Cattle	Cattle	≠	≠	=
11	Roum, NM C37412	Sheep	Sheep	Sheep/goat	=	=	=
12	Haraldskær, NM 3705	Cattle	*	Goat		≠	

''MO+LM'': macroscopical observation and light microscopy, ''LM+SEM'': light microscopy and scanning electron microscopy, ''MS'': Mass Spectrometry-based peptide sequencing. *This item is thought to be deliberately de-haired and only few hairs are preserved on the surface. Therefore there was only sufficient hair for one microscopic analysis. =/≠ indicate same/different species identification achieved by the methods compared.

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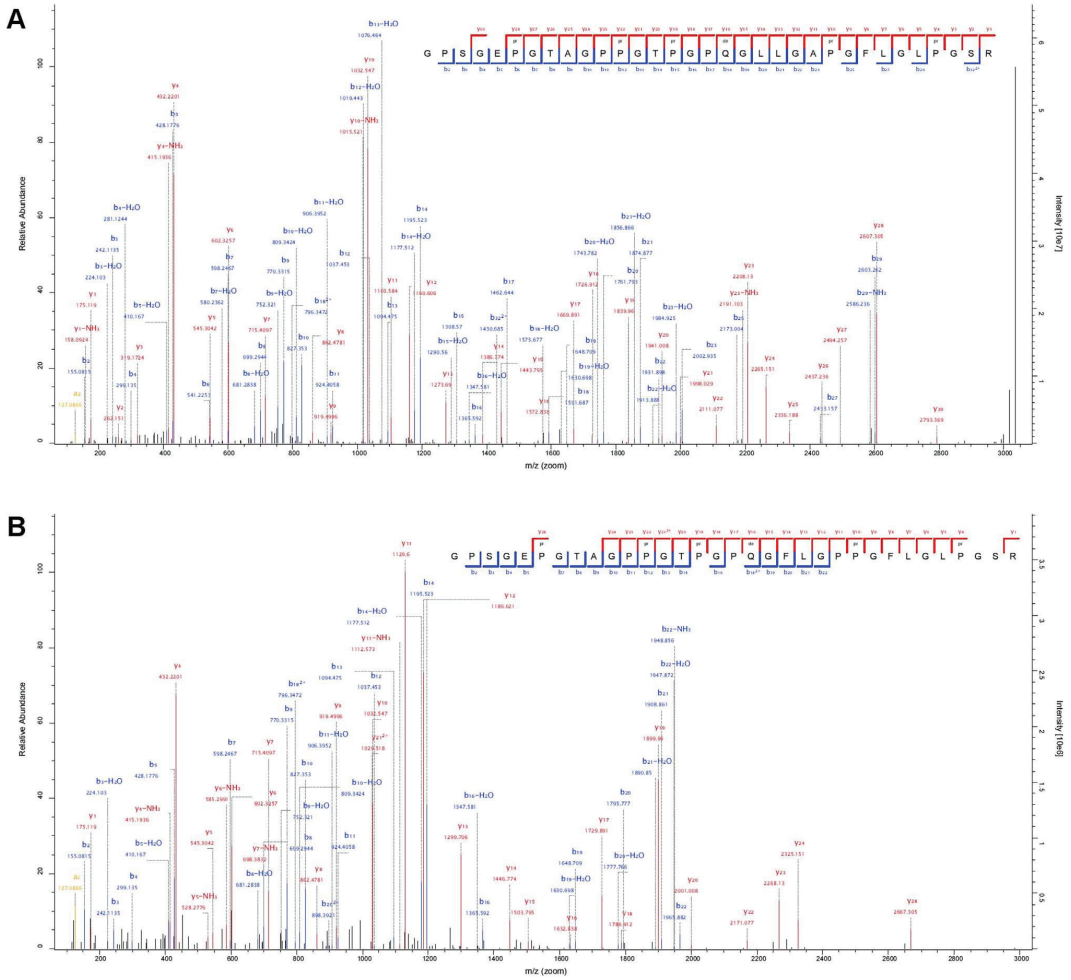

Figure 2. Examples of tandem MS spectra supporting identification of the type I alpha-2 collagen (COL1A2) sheep/goat diagnostic peptide [43,62]. A) MS/MS spectrum from the sample 9, from Karlby (D4854e), confidently assigned to amino acid sequence GPSGEPGTAGPPGTPGPQGLLGAPGFLGLPGSR, diagnostic for sheep. B) MS/MS spectrum from sample 8, (Karlby D4854c), confidently assigned to amino acid sequence GPSGEPGTAGPPGTPGPQGFLGPPGFLGLPGSR, diagnostic for goat.
doi:10.1371/journal.pone.0106875.g002

identification of sample 1 as goatskin, relies on several diagnostic peptides (Fig. S5 and Table S5 in File S1). The relevance of some of these peptides as a species marker has already been reported [63]. Based on the peptide signal, and the difficulties in microscopic identifications, the MS-based identification of the sample as goatskin is considered to be conclusive.

In addition, the availability of the cattle reference proteome and the recent public release of extended lists of sheep and goat proteins allowed for an exhaustive peptide and protein identification without limiting the search to a subset of the most abundant collagens and keratins [43,62]. Although publicly available protein lists currently used to assign peptide sequences to spectra are only complete, or significantly extended, for a relatively few mammal species, the number of mammal proteomes extensively covered is rapidly increasing. During the preparatory stage of our work, we observed, and took advantage of, the inclusion of extended protein lists for both sheep (*O. aries*) and goat (*C. hircus*) in public databases. This clearly demonstrates the rapid progress in this field. An example of the immediate implications of these contributions is that, until recently, the MS-based discrimination of the hairless skin and bone remains of sheep and goats was solely based on a single, relatively long, collagen peptide [43,62] (Fig. 2), now this discrimination can be achieved on the basis of a much longer list of species diagnostic peptides as reported for sample 1 and 12 (Table S3, S4 and S5 in File S1). This improvement is partially due to the experimental setup adopted, however, most of the previously unreported diagnostic peptides were detected adopting a shotgun proteomics approach instead of focusing on

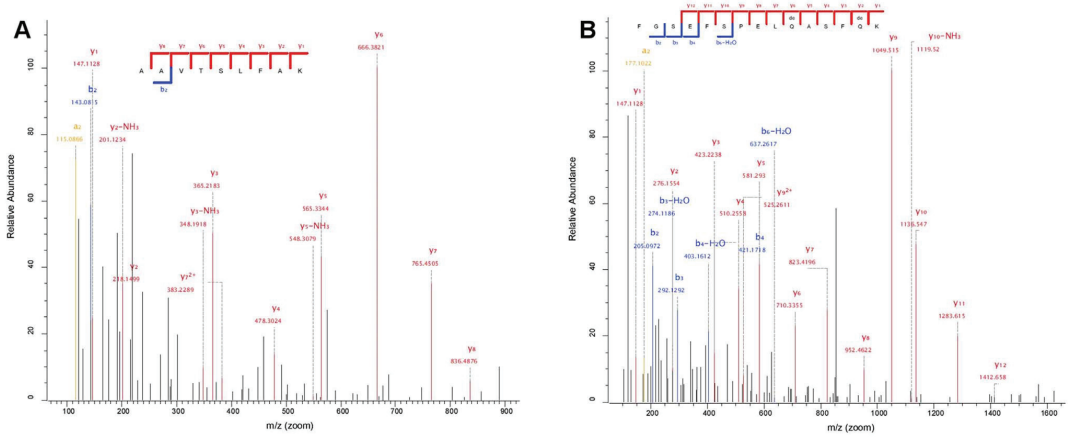

Figure 3. Tandem MS spectra from sample 10, Møgelmose supporting identification of bovine fetal hemoglobin subunit beta (UniProt accession number: P02081). MS/MS spectra confidently identified two peptide sequences: A) AAVTSLFAK and B) FGSEFSPELQASFQK.
doi:10.1371/journal.pone.0106875.g003

the few most abundant proteins in bone, skin and hair. The availability of more markers enables a more secure identification of goatskin samples, which are closely related to sheep and equally present in the same regions and time periods as the samples analysed.

Apart from improving species identification, the availability of a reference proteome, or an extended protein list, for the most common domestic animals further enables the identification of proteins solely expressed in a specific tissue, developmental phase, or biological process [54]. For example, the detection of foetal haemoglobin, which is only expressed in animals younger than a few months [64], suggests that the cape from Møgelmose, was produced from a calf slaughtered within a few months of birth. A skin element from this cape was previously identified as the genus *Martes* [11], thus the present analysis represents crucial new information, also on preferences for specific qualities for production, as calfskin is much softer than skins from older animals. At this slaughter age, skin and meat of a higher quality would have been obtained. This result adds new perspectives to the interpretation of prehistoric animal husbandry and is highly pertinent to broader studies of animal bone assemblages. This type of information can only be provided by protein analysis as, while the genome of an organism is almost identical in all its tissues and developmental phases, its proteome can be developmental phase-specific. Our results demonstrate that MS-based ancient peptide sequencing is a reliable method for species identification, and yields information unobtainable with other methods.

Despite the novel, reliable results providing secure species assignment, the identification of archaeological skin garments based on MS-based ancient peptide sequencing also comes with certain limitations. In particular, reference protein databases are still incomplete, as exhaustive protein lists are at the moment only available for a limited number of species. This shortcoming, however, will eventually become less of an issue in the near future, as the rapid progress of genome-sequencing projects will soon make reference proteomes available for an increasing number of species, enabling even more secure species identification and higher taxonomical resolution. MS-analysis remains a (minimally) destructive approach, requiring sophisticated equipment and laboratory facilities. Consequently, it cannot be immediately available for all archaeological skin samples, and its diagnostic value is limited to the analysed skin element, which is only one among the many elements used to assemble a skin garment.

Although PMF-based approaches allow relatively rapid and inexpensive characterisation, thus making this approach ideal for large-scale applications and commercial quality control analyses, the maximisation of molecular recovery and data interpretation is crucial when applying even minimally destructive analyses to irreplaceable material of high cultural heritage value. Despite the necessity to sacrifice small parts of archaeological objects in the process, the collection and public sharing of the richest possible set of molecular information compatible with the technology and knowledge available at the time of analysis is of infinite value for the understanding of our distant past.

Conclusions

The aim of this study was to compare established, morphology-based methods for species identification of archaeological skin objects from bogs with MS-based ancient peptide sequencing. The three methods adopted, in some cases, gave inconsistent results. Microscopy was challenged by general problems caused by degraded and partial fibres of archaeological material, while MS yielded secure peptide signals indicating that this method is suitable for application in the archaeological context examined. It thus represents a promising approach for future archaeological skin garment species identification. Although public databases of protein sequences are not yet complete, they already enable the determination of the most common domesticated species. Microscopy, on the other hand, holds the advantage of being relatively inexpensive, non-destructive, and easily applicable to a large number of samples, and sometimes, the sole option when dealing with mineralised samples. MS-based peptide sequencing could also be used to improve microscopy-based identification through the creation of reference collections of archaeological skin samples securely identified by peptide sequencing validation.

Based on the results presented here, it may be concluded that morphology-based species identification methods represent valid preliminary screening tools; however, for de-haired samples, or samples assigned by microscopy to species other than sheep, mass

spectrometry-based peptide sequencing is highly recommended for achieving secure species identification.

Supporting Information

File S1 Supporting Information file containing supporting text, figures and tables.
(PDF)

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Author Contributions

Conceived and designed the experiments: LØB ALS UM EC. Performed the experiments: LØB ALS CDK EC. Analyzed the data: LØB ALS CDK MS EC. Contributed reagents/materials/analysis tools: ALS CDK JVO EC. Contributed to the writing of the manuscript: LØB ALS UM EC.

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3. Species identification of skins from Arctic costumes – study in progress

Introduction

The National Museum of Denmark has a unique collection of historic skin costumes from the circumpolar area. It comprises clothing of the Inuit, Yupiit and Aleut in Greenland, Canada and Alaska, as well as the Sami of North Scandinavia and Siberian people in Russia (Fig. 31). The costumes were collected between 1800 and 1950 by expeditions to the Arctic regions.

This collection is the focus of the research project *Skin Clothing from the North* directed by Anne Lisbeth Schmidt, which is part of the large-scale strategic priority *Northern Worlds* of the National Museum of Denmark (Schmidt 2010, 2012).

The major purpose of *Skin Clothing from the North* is to investigate possible connections between skin types, manufacture, sewing and design. This information is integrated in a wider analysis of circumpolar peoples' lifestyles, geographical alliances, interaction and the influence of trade. The identification of species is therefore an important contribution to the project. One of the project aims was to create a model to describe skin costumes by their design, stitching and the animal species of the skins used. The latter has hitherto not been performed systematically, but only by random visual inspection. During this project the skins were species identified by light microscopy and as demonstrated here by DNA analysis, performed by the author.

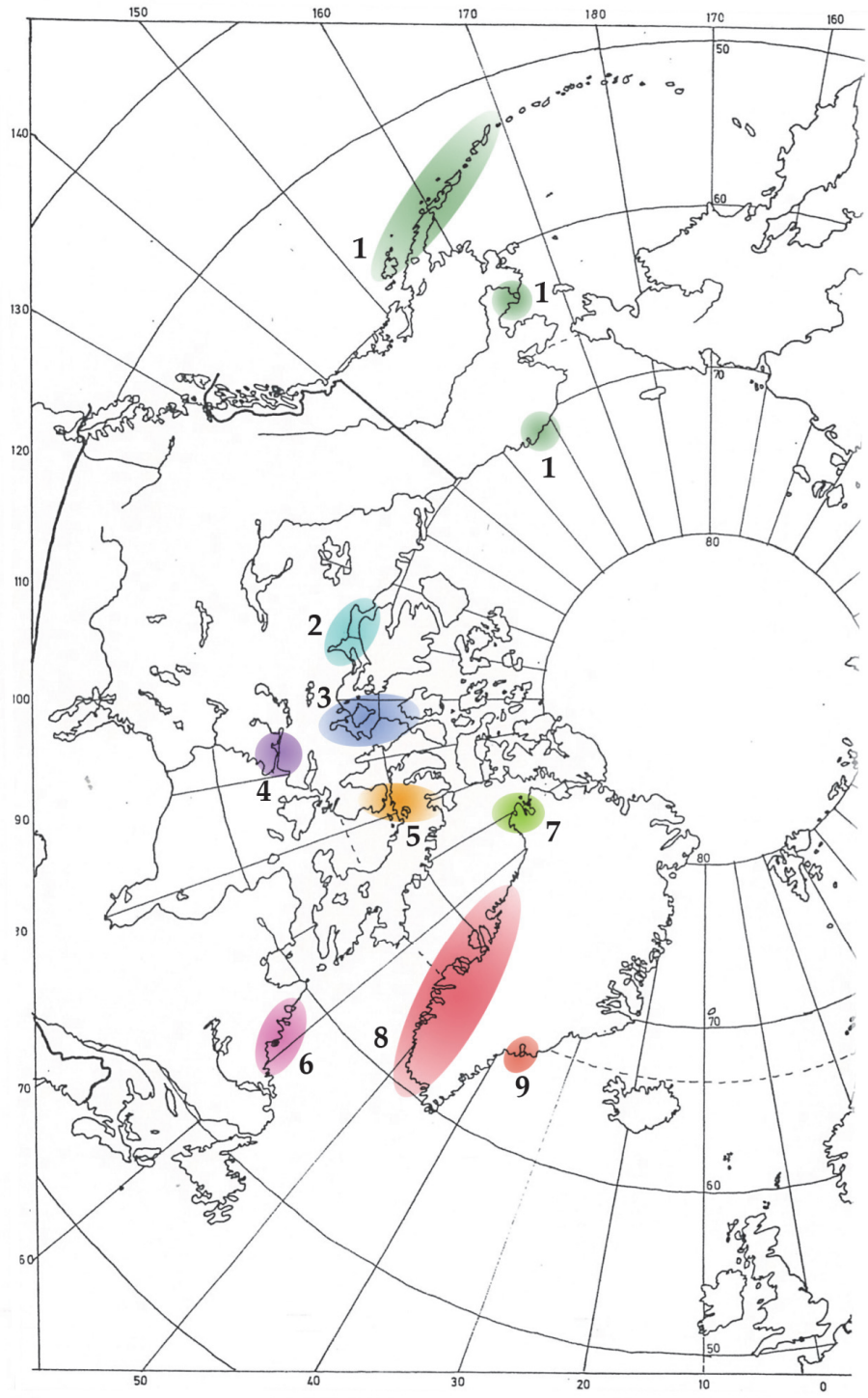

Figure 31. Map of Arctic people. 1. Inuit, Alaska. 2. Copper inuit 3. Netsilik inuit 4. Reindeer inuit 5. Iglulik Inuit 6. Inuit, Labrador 7. Polar Inuit 8. Inuit, West Greenland. 9. Inuit, East Greenland. Graphics: Sidsel Frisch.

Material and method

We received samples of 75 costume parts. The samples consisted of skin, hair or both. Skin samples measured in average 0,5x0,5 (Fig. 32).

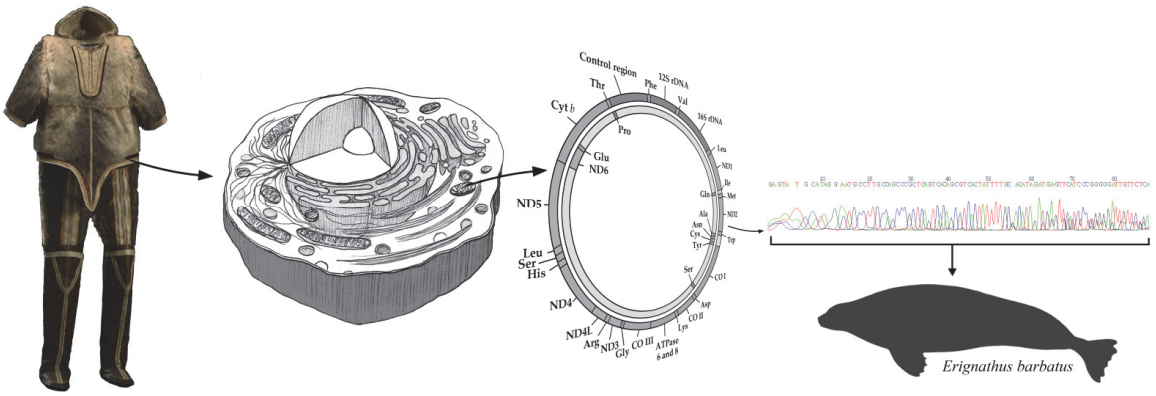

Figure 32. Flow diagram for extraction and sequencing of DNA from Arctic costumes. Graphics: Sidsel Frisch.

DNA extraction

DNA was extracted from all samples following Gilbert et al. (Gilbert et al. 2004) with slight modifications. Before digestion, the samples were washed in 5-10% bleach, rinsed with water, and then digested in 500µL of buffer at 55 degrees on rotor over night. The next day, another 50µL of proteinase K was added for additional incubation for another night. The samples were purified using the Quiaquick Qiagen purification kit and eluted in 30µL of EB buffer.

Genetic analyses

Extracted DNA was analysed using a PCR-assay, targeting a mammalian 'barcoding' fragment 16S region of the mitochondrial genome. Initially the primerset 16SA&M (Rasmussen et al. 2009 with additional tag) were used to amplify an approximate 122 bp long amplicon (including primers). As Sanger sequence data usually has low quality at the beginning and end, an additional extension of the amplicons were performed with the extension primers Fusion_extended_F&R to allow the sequence data of the middle section to be of high quality.

Primer name	Amplicon length	Sequence	T_a (°C)
16SA&MF 16SA&MR	122 bp (varies after species)	5'-GCC TTG CCA GCC CGC TCA GCG CTG TGA TCA CTA TTT TGC NAC ATA GA-3′ 5'-GCC TCC CTC GCG CCA TCA GTC ACA GCG CCC CGA AAC CAG ACG AGC TA-3′	54
Fusion_extended_F Fusion_extended_R	261 bp	5′-GTA AAA CGA CGG CCA GCC CTT GAC TAC TGA AGT ACA GTA TCC AGA CGC CTC CCT CGC GCC ATC AG-3′ 5′-CAG GAA ACA GCT ATG ACT CAA TAA GCT GCA ATT ATG CCA TAG GAC ATG CCT TGC CAG CCC GCT CAG-3′	54

Table 9. Primers used for species identification of Arctic costumes.

Each 16s and fusion 25-µl PCR reaction consisted of 0.1 µl Amplitaq Gold (ABI, Foster City, CA), 1× Amplitaq Gold buffer, 2.5 mM MgCl, 400 nM each primer, 25nM each dntp, and 1 µl DNA. Cycling parameters were as follows: enzyme activation 95°C for 10 min, 40 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 30 s, and a final extension of 7 min at 72°C.

Purified products were Sanger sequenced using the commercial service offered by Macrogen Ltd (Amsterdam, Netherlands).

Data analysis

The forward and the reverse sequence were assembled along with the 16SA&M primers in Geneious Pro 4.7.6 (Biomatters Ltd). The 29 bp product between the primers was checked carefully to see if the sequencing machine had called the nucleotides correctly. This was done by visually inspecting the chromatograms for clearly erroneous base calls, at which the sequencing machine was not able to precisely determine the nucleotide represented. This may be due to overlaying ambiguous peaks or broad peaks. These can often be called manually by evaluating the nucleotide signal strength and spacing. When the 29 bp of sequence was confirmed it was blasted against the NCBI Genbank database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

54 of the 75 analysed samples yielded amplifiable DNA. Of these, two gave a 100% match with human DNA which must be interpreted as contamination, which seems likely after up to two centuries of handling. Two samples had sequences of too inferior quality to analyse and one sample did not yield any 100% matches. However, 49 samples yielded 100% matches with one or more species/genera found in the circumpolar area (see Table 10).

Object ID	Area	Gender and sample Name	Type of Garment	Species
30492	West Greenland	Woman 3 Lc.187a	Parka	Several species of the family *Cervidae**
32996	West Greenland	Boy 1 L.9353	Parka	*Phoca*
32995	West Greenland	Boy 1 L. 9352	Trousers	*Phoca*
31316	West Greenland	Woman 2 Ld32b	Trousers	*Phoca*
31317	West Greenland	Woman 2 Ld32c	Boots	*Phoca*
31313	West Greenland	Man 1 Ld.31b	Trousers	*Phoca*
32086	East Greenland	Man 2 L.4990	Parka	*Phoca*
31432	East Greenland	Woman 2 Ld.133.9	Trousers	*Phoca*
31535	East Greenland	Woman 2 L.1545.2	Boots	*Ursininae* (polar bear or brown bear)
31299	East Greenland	Man 1 Ld.17	Parka	*Erignathus barbatus*
32179	East Greenland	Woman 1 L.5082	Boots	*Canis lupus***
33005	Polar	Man 1 L.9549	Parka	*Canis lupus* or *Vulpes vulpes*
33006	Polar	Man 1 L.9551	Boots	*Erignathus barbatus*
33009	Polar	Woman 2 L.9554	Parka	*Phoca*
33010	Polar	Woman 2 L.9555	Trousers	*Canis lupus* or *Vulpes vulpes*
33011	Polar	Woman 2 L.9556	Boots	*Phoca*
34035	Polar	Woman 1 x.233	Parka	*Canis lupus* or *Vulpes vulpes*
32950	Polar	Woman 1 L.9271	Boots	*Ursininae* (polar bear or brown bear)
36417	Labrador	Woman 1 P.230b	Trousers	*Phoca*
36423	Labrador	Woman 1 P.231c	Boots	*Phoca*
69944	Labrador	Man 1 P26.18	Parka	*Canis lupus***
69971	Labrador	Man 1 P26.19	Trousers	*Canis lupus***
69942	Labrador	Man 1 P26.20	Boots	i
34595	Iglulik	Woman 1 P27.454	Trousers	Several species of the family *Cervidae**
34598	Iglulik	Woman 1 P27.457	Boots	Several species of the family *Cervidae**
34549	Iglulik	Man 1 P27.410	Parka	Several species of the family *Cervidae**
34579	Iglulik	Man 1 P27.439	Boots	*Phoca*
34945	Reindeer	Man 1 P28.6	Inner parka	Several species of the family *Cervidae**
34953	Reindeer	Man 1 P28.14	Trousers	Several species of the family *Cervidae**
34970	Reindeer	Man 1 P28.31a-b	Boots	*Phoca*
34949	Reindeer	Woman 1 P28.10	Parka	Several species of the family *Cervidae**
34957	Reindeer	Woman 1 P28.18	Trousers	Several species of the family *Cervidae**
34973	Reindeer	Woman 1 P28.34a-b	Boots	Several species of the family *Cervidae**
37357	Kobber	Woman 1 P30.15	Parka	Several species of the family *Cervidae**
37374	Kobber	Woman 1 P30.30	Trousers	Several species of the family *Cervidae**
37388	Kobber	Woman 1 P30.43a-b	Boots	*Phoca*

Object ID	Area	Gender and sample Name	Type of Garment	Species
37369	Kobber	Man 1 P30.25	Trousers	Several species of the family *Cervidae**
37387	Kobber	Man 1 P30.42a-b	Boots	*Phoca*
35236	Netsilik	Man 1 P29.8	Inner parka	*Phoca*
35425	Netsilik	Man 1 P29.17	Trousers	*Ovibos moschatus*
35238	Netsilik	Woman 1 P29.10	Parka	Several species of the family *Cervidae**
35246	Netsilik	Woman 1 P29.18	Trousers	Several species of the family *Cervidae**
35263	Netsilik	Woman 1 P29.28a-d	Boots	Several species of the family *Cervidae**
36654	Alaska	Man 1 P.1677	Boots	*Phoca*
36958	Alaska	Woman 1 P32.1	Parka	*Canis lupus***
36968	Alaska	Woman 1 P32.11a-b	Boots	*Phoca*
36961	Alaska	Man 2 P32.4	Trousers	*Phoca*
36682	Alaska	Boy 1 P.6403	Parka	*Gulo gulo*

Table 10. Succesful samples with species assignments.

Of the succesful samples, some could be assigned to a single species, others matched several species. That the samples could not be assigned further is due to the short sequence of DNA that we work with (29 basepairs). Due to this, there may be cases in which the amplified sequence does not display differences between species and therefore matches two or more species 100%. In such cases, one must evaluate which result is the most likely. This is the case for *Phoca* (seals), *Cervidae* (deer), *Ursininae* (Bears), and *Canis/Vulpes* (wolf/dog/fox).

Seals

Among seals, *Erignathus barbatus* is distinguishable from the species naturally found in the Arctic (see Table 11). The other species are not possible to tell apart based on the sequence analysed.

Latin	English
Phoca vitulina	Harbour seal
Pusa hispida	Ringed seal
Erignathus barbatus	Bearded seal
Cystophora cristata	Hooded seal
Pagophilus groenlandica	Harp seal
Halichoerus grypus	Grey seal

Table 11. Seal species in the Arctic area.

Cervidae

For the samples referred to the family *Cervidae*, several species matched the sequences 100%. Of these species, all except *Cervus elaphus* (Red deer) and *Rangifer tarandus* (Reindeer) live outside the circumpolar area (Africa and Southern Asia). It was therefore assumed that the samples should be assigned to one of the two first species. For the sample from Greenland, reindeer seems to be the only option. North America also seems to be outside the area of distribution of reindeer. The distribution may have changed over time, but it is not very likely that it has changed dramatically within the last 100 years. Species should therefore not immediately be excluded based on the modern distribution. An analysis such as this may be used to demonstrate the presence of species in historic and prehistoric times.

Canis/Vulpes

In the case of *Canis* and *Vulpes*, there are two scenarios seen in the material. In four cases, marked with ** in table 10, the sample was determined as *Canis lupus*. In this case, it is not possible to distinguish between *Canis lupus familiaris* (domestic dog) and *Canis lupus lupus* (wolf), as these are very closely related. In three cases, *Canis lupus* and *Vulpes vulpes* (fox) cannot be distinguished. This means that both domestic dog, wolf and fox are possible candidates for the species for these samples. Possibly, fox will be distinguishable from dog and wolf by the colour of the fur while this is probably more problematic for the two latter species.

Ursininae

Several species turned up as possible candidates in this group, as both polar bear and brown bears match the samples 100%. Between these species, polar bear should be possible to distinguish from the others based on the colour of the fur. However, at a seminar (26-27 November 2009) for the research project Dragter fra Nordlige Verdener, it turned out that polar bear was hard to distinguish from dog. These two species are however unproblematic to distinguish using DNA analysis.

Discussion and conclusion

Of the 75 analysed samples, 21 did not yield amplifiable DNA. This probably means that either there was no DNA left in the samples or that the remaining fragments were to short to be extracted with the primerset used. It is possible that some of these may yield amplifiable DNA using methods that are able to target shorter DNA fragments as Illumina library construction and sequencing (see chapter 1 and method section).

Hair samples from museums have previously been demonstrated to provide a good material for DNA analysis even after long storing at room temperature (Gilbert et al. 2007). As the samples are at the maximum 200 years old and were never buried, they were suspected to yield a good successrate. However, 28% of the samples still did not yield amplifiable DNA.
There is no clear evidence that samples from the same Arctic regions perform better than others, or that certain costume parts provide better results than others. As the different costume parts require different preparation of the skin, the preservation of DNA in some parts rather than other could have implied that preparation of skin including tanning processes could have an effect on DNA preservation. Water skin used for boots for instance require a longer tanning in urin than the regular skins for garments. This does not, however, seem to be the case (Hart Hansen et al. 1991: 122-124).
As for the conservation of the costumes from the greenlandic mummies from Qilakitsoq, the costumes have been tanned with alun to be able to unfold them (Hart Hansen et al. 1991: 132-133). The tanning agent alun has been proved to act degrading on DNA (Brandt et al. 2011). The conservation and re-tanning of the costumes would therefore decrease the chances of retrieving DNA from them. I have no knowledge of such conservation processes, which however does not mean that thay have not taken place.

As the research project is not finished, the results of the DNA analysis have not yet been compared with the microscopic analysis performed by project leader Anne Lisbeth Schmidt. This will be one of the next steps in the finalising of *Skin Clothing from the North*. There are therefore no conclusions on correspondence or disagreements between the methods. As the species analysed in this study (seals, dog/wolf, dears and bears) are more distantly related than the species analysed in chapter 2 (sheep, goat, and cattle) their hairs may differ more and are easier to distinguish by hair microscopy on family level. Distinguishing on species level is be more difficult. Distinguishing to species level within the seals, bears, cervids and dog/wolf is therefore also the cases in which DNA analysis is most relevant.

This is moreover interesting, as different seal species are known historically to be used for different parts of the costume (pers. com. Anne Lisbeth Schmidt). It would thus be interesting to see if this is also the case for this collection. Moreover, it would provide information about past distribution of seals. The decrease of seals around East Greenlandic Ammassalik in 19th century caused starvation. This is reported by Gustav Holm who visited the settlement in 1884 (Gulløv 2004: 340-343). Gustav Holm's acquired collection from Ammassalik including skin costumes could for instance provide evidence of seal species present at the time of starvation.

According to conservator and project leader Anne Lisbeth Schmidt, it is often possible morphologically to identify the seal species by the bare eye, as the skins of different species have easily recognisable patterns of rings. Also the cross sections of the hairs appear quite distinct. As the present analysis was not able to distinguish seal species apart from *Erignathus barbatus*, the relation between microscopic species identification of hairs have not been confirmed by species identification by DNA analysis. This would be useful to verify the microscopic method, though the samples in the present study are younger than the samples presented in chapter 2 and therefore less likely to display evolutionary differences as seen in for instance prehistoric sheep (see chapter 1 and 2). Species identification of seals by DNA would require an expanded analysis including additional regions of DNA that differs between the seal species. Several studies distinguishing seal species have already been published (Stanley et al. 1996, Davis et al. 2004, Arnason et al. 2006, Fulton and Strobeck 2009, 2010, Masland et al. 2010). Some attempts to species identify seal skins from the collections have already been made. Initial attempts of traditional PCR and following Sanger sequencing (pers. com. Jesper Stendetup, Centre for GeoGenetics) were not succesful, perhaps because the targeted regions were too long (three of four tested primersets targeted more than 300 bp). Species identification may however be possible targeting shorter sequences and using more advanced methods as for Illumina library build and sequencing of the entire mitochondria (see method section and chapter 1).
The verification of microscopic hair identifications of for instance hairs for different seal species would provide archaeologists with a less expensive and time consuming and more available tool for species identification.

The ongoing studies will reveal if the species identification by DNA analysis presented here corresponds with the identification performed by microscopy.

Introduction

The hitherto oldest remains of skin elements in the Arctic region derive from the West Greenlandic Saqqaq culture settlement of Qeqertasussuk. C^{14} datings of the layers in the settlement midden has shown that it was in use from 2370-2425 cal. BC to 1515-1335 cal. BC (Grønnow 1990: 28). The skin remains seem to adhere to the time around 2000 BC (Grønnow 1990: 35). Remains of skin material are extremely rare in archaeological contexts as their preservation requires particular environments. At Qeqertasussuk, skin fragments were preserved in the permafrozen culture layers of the midden and in the dwelling area (Grønnow 1990: 35). These rare skin fragments provide us with a unique opportunity to study the sewing- and costume tradition of the first humans (paleo eskimos) in Greenland, and whether this tradition was carried on to the techniques of the more well known costumes of the Thule culture (AD 1200-1900 (Gulløv 2004).

An interesting aspect of the skin fragments is their testimony of the animal species utilized for the production of costumes and other skin products such as tents and straps. In the later Thule culture, these products were produced from different species of seal. Whether this was also case in the Saqqaq culture is uncertain. Moreover species identification of the skins would provide us with additional knowledge on the utilization of animal resources, adding to that which can be inferred from studies of faunal remains. Due to the good preservation status of the skin fragments, hair and feathers are often preserved on the skin surface. A previous study has used these elements for identification of the species to caribou, dog, seal and bird. This was published in Danish by conservator Gerda Møller (Møller 1991), see Table 12. However, the species of seals and birds could not be determined. This information is however relevant for the understanding of the exploitation of animals at the site and the skin production tradition.

Several new scientific methods including the analysis of ancient DNA and proteins have been applied in recent years for species identification of archaeological materials (Schlumbaum 2010, Hollemeyer 2008, 2012, Toniolo 2012, Kirby 2013), and have proved valuable as a supplement to microscopic and macroscopic species identifications of animal skins (Schmidt et al. 2011, Brandt et al. 2014). The success of ancient DNA analysis, however, relies on the environment in which the samples were buried. Permafrozen environments are known to be among the best for the preservation of DNA (Smith et al. 2001, Willerslev, Hansen and Poinar 2004). In 2010, a tuft of human hair from Qeqertasussuk yielded the first genome of an ancient human (Rasmussen et al. 2010). This result gave good expectations for analysing ancient DNA from the skin fragments from the same site.

This study explores if the species identity of the skins can be resolved by analysing their DNA by PCR amplification and sequencing.

Materials and methods

Seven skin samples from Qeqertasussuk were analysed (see Table 12). The samples were provided by the National Museum of Denmark.

No.	Museum numbers	Description	Species identification (Møller 1991)	Species identification Schmidt
1	Qt 87 20/19:92	Skin sample from upper of inner stocking	Seal	
2	Qt 87 20/19:46 fragment B	Skin sample from tent fragment	Seal	
3	Qt 87 20/19:46 no. 1	Skin sample from strap	Seal	
4	Qt 87 Structure A8 hair from layer 2 between section E and D	Hair sample from leather bag	Reindeer	
5	Qt 87 20/19:46 fragment A	Thread sample from tent seam	Reindeer/fin whale	
6	Qt 87 20/19:46 no. 5	Skin sample from fragment	Bird skin	
7	Qt 87 20/19:46 no. 4	Skin sample from fragment	Dog?	Artic hare

Table 12. The seven samples analysed from Qeqertasussuk.

DNA extraction and contamination control measures

Archaeological materials are in general expected to contain relatively low levels of endogenous DNA, and are thus at high risk of contamination from external sources of DNA. Therefore, strict protocols must be followed to minimize the risk of contamination of the material during the analyses. To comply with these precautions, all analyses were performed in dedicated ancient DNA extraction laboratories, isolated from laboratories used for studies of modern DNA (including PCR amplicons). All reagents used were molecular biology grade, and all extractions and subsequent PCR analyses included blanks to monitor for contamination.

DNA was extracted from all samples following Gilbert et al. (Gilbert et al. 2004) with slight modifications. Before digestion, the samples were washed in bleach and rinsed with water and then

digested in 500µL of buffer at 55° degrees on rotor over night. Subsequently, another 50µL of proteinase K was added for additional incubation for another night. The samples were purified using the Quiaquick (Qiagen, Valencia, CA) purification kit following the manufacturer's instructions, and eluted in 30µL of EB buffer.

Genetic analyses

Post-DNA extraction, the small fragments of mitochondrial DNA were analysed using a PCR-assay, targeting the 16S (for mammals) and 12S (for birds) regions of the mitochondrial genome (see Fig. 6, method section). Primers used were mammal (Tagged mammal primers, 16SA og 16SM) and bird (Aves_12Sa, Aves12S_c (Epp et al. 2012) generic (see Table 13). The mtDNA primers used amplify a respectively around 122 bp and 52 bp long fragment of the mitochondrial 16S gene, the sequence of which is diagnostic to respectively mammals and birds.

Taxon	Primer name	Amplicon length	Sequence	T_a (°C)
Mammalia	16SA&MF 16SA&MR	Ca. 122 bp (varies by species)	5'-GCC TTG CCA GCC CGC TCA GCG CTG TGA TCA CTA TTT TGC NAC ATA GA-3′ 5'-GCC TCC CTC GCG CCA TCA GTC ACA GCG CCC CGA AAC CAG ACG AGC TA-3′	54
Aves	Aves_12Sa Aves_12Sc	52 bp	GATTAGATACCCCACTATGC GTTTTAAGCGTTTGTGCTCG	58

Table 13. Primers used for species identification of skin samples from Qeqertasussuk.

Each 16s 25-µl PCR reaction consisted of 0.1 µl Amplitaq Gold (ABI, Foster City, CA), 1× Amplitaq Gold buffer, 2.5 mM MgCl, 400 nM each primer, 100 nM each dntp, and 1 µl DNA. Cycling parameters were as follows: enzyme activation 95°C for 10 min, 40 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 30 s, and a final extension of 7 min at 72°C.

Each 12s 25-µl PCR reaction consisted of 0.2 µl Platinums Taq High Fidelity DNA Polymerase (Invitrogen, Carlsbad, CA), 1× PCR buffer, 1 mM $MgSO_4$, 400 nM each primer, 200 nM each dntp, and 1 µl DNA. Cycling parameters were as follows: enzyme activation 95°C for 10 min, 40 cycles of 95°C for 30 s, 58°C for 30 s, and , 72°C for 30 s, and a final extension of 7 min at 72°C.
Post-PCR, amplicons were viewed on 2% agarose gels.

Results

No samples analyzed yielded any amplifiable DNA. A spike test of the samples showed that they were inhibited, but even after dilution and repurification removing inhibitors, the samples gave no indications of DNA.

Discussion and conclusion

Despite the good expectations for this material derived by the results tuft of human hair found at the same site (Rasmussen et al. 2010), the samples failed to yield amplifiable DNA. The reasons for this disappointing result could be explained by for instance the use of preservatives and second the amount of material used for analysis.

Schmidt notes that sample 1 and 2 may be full of the preservative PEG (polyethylene glycol) and glued with PVA (polyvinyl acetate), whereas preservatives should not have been used on sample 3 -7. Apart from the risk of DNA contamination derived from animal-based glues (Nicholson et al. 2002), preservatives have been demonstrated to have a damaging effect on the preservation of DNA in museum samples (Pruvost et al. 2007, Eklund and Thomas 2010). Preservatives can not be ruled out as part of the explanation why sample 1 and 2 did not yield DNA. Future samples should be taken from specimens or areas of objects known not to have been exposed to preservation treatment. The other five samples which have not been treated did however not work either. This could point to issues of more general preservation conditions on the site or sampling size.

The permafrozen environment should be optimal for DNA survival as demonstrated by Rasmussen et al. 2010, though not all samples from permafrozen environments have yielded DNA (Campos et al. 2010). The size of the skin samples may however have something to say, as some of the samples were very small. From sample five just few hairs were recieved. On the contrary the sample size of the Saqqaq hair tuft was approximately 10 x 15 cm of which three samples of 1,5-2 grams were digested. This provides the skin samples with a much smaller chance of yielding DNA compared to the hair tuft. Also another more sensitive method including Illumina library build was applied for the hair tuft.

Species identification by PCR and sequencing DNA of the samples was not possible. The negative results of the analysis does not, however, nessecarily mean that there is no DNA left in the samples or that they cannot be analysed with a different method. Other options could therefore be considered.

As species identification was the goal, other types of analysis offering lower resolution but still

providing the species identification could be applied. As seen earlier, ancient proteins may survive in materials in which ancient DNA is highly degraded (Brandt et al. 2014, see method section).

ZooMS (ZooArchaeology by Mass Spectrometry) has become very popular for species identification in archaeology. ZooMS takes advantage of the slow evolution of collagen, which means that species have a "molecular barcode" enabling them to be distinguished by their collagen. Methodologically, ZooMS uses the well established PMF (Peptide Mass Fingerprinting) combined with high throughput Time of Flight Mass Spectronomy (see method section). Samples are species identified by differences in the mass of the peptides, which are results of sequence differences between species.

The skin fragments from Qeqertasussuk are however small and have been tested several times. Therefore additional destructive testing of the priceless samples was not desirable.

A less destructive sampling technique for leather has been developed at BioArCh, Department of Archaeology, University of York. The methods allows species identification by analysing erdu created by rubbing erasers against a material.

At this timepoint however, the discrimination of seals using this method is restricted to distinguishing Phocaids (earless seals) from Otariids (sealions and fur seals) and Odebenidae (whalrus). Moreover, birds have not been explored so far. For this material in which precisely these species were of interest, the question is at this point in time not possible to solve using this method.

An alternate, DNA-based, option would be to construct Illumina sequencing libraries on the extracts (see method section) and subsequently sequence these, as this allows for amplification of shorter products that may still be present in the skins but are too short to be amplified with the primers used.

Being unable to apply the less destructive sampling by the method used by Bioarch, we decided that further sampling of the skin fragments should wait for future analysis and further developed methods providing new possibilities. There was no guarantee that there would be preserved DNA, so the sampling for this analysis was rejected. Though it would have been interesting to include species identifications of the skins in Grønnow's publication of the Qeqertasussuk, the decision of leaving material for future analysis is not limited to this study.

As the amount of material needed for destructive methods is decreasing as demonstrated by Bioarch, this provides good perspectives for future species identification. However, this also requires that and databases include more material for comparison.

PART III

Conclusions

Archaeological conclusions and future methodological perspectives

The primary aim of this PhD thesis was

1) to investigate sheep wool development by combining archaeology, textile research and next generation sequencing of sheep DNA and

2) to species identify skins using PCR and MS-based peptide sequencing.

The first study presented in part II deals with the earliest developments of sheep wool, which led to a fibre quality that could be used for textile production and its further development. The archaeological conclusions from my study are:

1) Judging from the archaeological evidence, the prehistoric costume and the technologies used for its production seem to undergo significant changes in the Danish Late Neolithic Period. This phenomenon is likely to be linked to the development and introduction of the wool fibre as a new raw material for textile production.

Sheep were present in Denmark from around 4000 BC. At this time, several technologies to produce textiles from plant fibres were already in use and well-known. Weaving, however, seems to be introduced in the Late Neolithic when loom weights begin to appear in the archaeological record. Changes in jewellery style and the introduction of dress pins also indicate costume changes at this time. In my opinion, a plausible explanation is that these changes coincide with the introduction of a new fibre for textile production: wool, since wool was already utilised in the Únětice culture with which Scandinavia had close contacts. That wool has not been documented in the Late Neolithic could be explained by the burial customs of this period, which are less likely to preserve organic materials than the oak coffins of the Early Bronze Age.

The attestation of loom weights demonstrate that a local production of woven textiles took place. Sheep, however, do not make up a large part of the zoological material and, although increasing in frequency throughout the Bronze Age, it is not until the Pre Roman Iron Age that kill-off-patterns demonstrate a more targeted production of wool (Kveiborg 2008). The raises the question whether the textiles of the Early Bronze Age were locally produced, whether they were made from local wool, and whether this was also the case for even earlier textiles. Scandinavian Bronze Age textiles have a very distinct, and recognisable appearance (Mannering in prep.b). Their uniformity suggests that the textile production was local. Future strontium isotope analysis will be useful in order to clarify if also the wool was local. However, strontium isotope analysis of a textile from Voldtofte and the Huldremose II garment dating to respectively the Late Bronze Age and the Pre Roman Iron Age have demonstrated that both plant fibres, wool or sheep could in fact be imported (Frei et al. 2009b, Bergfjord et al. 2012).

2) Based on comprehensive measurements of the wool fibre diameter conducted on textiles from Denmark, a second significant change in the wool fibre becomes visible in the Roman Iron Age textiles (AD 1-400).

Yet, already in the Late Pre Roman Iron Age (500-1 BC), iron shears come into use in Southern Scandinavia, indicating that sheep wool is no longer of the primitive non-shedding type. In my opinion, this is plausibly related to the changes in sheep wool fibre diameter attested in the Roman Iron Age indicated by new wool fibre analysis. We can therefore, based on the archaeological material conclude that sheep wool in Denmark changed around the Common Era .

This could be explained by the increasing contacts with continental Europe, which already had different sheep types earlier in the first millennium BC. In the Roman Iron Age, the costume traditions change, the use of dress accessories increases, and the agriculture undergoes important changes such as the introduction of new species; these changes are most probably due to contacts with the South.

My study thus suggests that two major changes of the wool fibre took place in the Danish prehistory, one in the Late Neolithic and one in the Roman Iron Age. Whether these changes represent a local, isolated development, or reflect the introduction of a different sheep type or simply the import of a new wool type, is still unclear.

Due to the generally low contents of endogenous DNA in the samples, we can conclude that the technology applied in the present study of ancient Danish sheep was unable to add notable information to the genetic development of sheep and their wool in the Danish archaeological material. The study, however, outlined potentials and clarified limitations that should be taken into consideration in future investigations of wool development:

1) DNA could not be recovered from any of the ancient textile samples included here; however, the feasibility to recover DNA from wool has previously been demonstrated (Brandt et al., 2011). The retrieval of DNA is hampered by the environments in which ancient textiles are preserved in Denmark, in the Bronze Age oak coffins and the peat bogs. Inhumation graves, too, proved to be more degrading environments for DNA preservation than anticipated. I would expect that samples of younger age from, for instance, the Viking period, Medieval or later times, and samples from colder environments, such as those recovered in Greenland, or alkaline burial environments currently present the best options and opportunities for future research on genetic characteristics of ancient wool textiles.

2) The analyzed sheep bones provided two almost complete mitochondrial genomes. The targeted nuclear markers could, however, not be retrieved from any of the included bone samples. Although the cool environments of northern Europe are generally believed to present favourable preservation

conditions for ancient DNA, many factors are in play and determine the preservation of endogenous DNA. Despite the fact that these factors were carefully considered in the sample selection phase, still, none of the included samples demonstrated excellent preservation of DNA. Based on the samples included in this PhD thesis and the contexts from where they were recovered, I can conclude that sheep bones represent a superior source material over wool in order to perform a study of prehistoric sheep in Denmark.

3) Despite the low resolution of the sheep mitochondrial genome, the analysis of additional ancient sheep mitochondrial genomes could still contribute more valuable data, which could potentially clarify the prehistoric immigration of sheep and the emergence of new sheep breeds in southern Scandinavia.

4) Nuclear markers currently provide the best options for addressing and resolving questions of genetic traits and population genetic questions. Therefore, nuclear DNA markers should be the focus of future research on ancient sheep DNA. The study of nuclear DNA markers would, however, require a selection of samples with a higher content of endogenous DNA.

The results and methodological recommendations deriving from the present study suggest the following focus areas for future analyses of sheep wool development:

1) Optimizing the sampling strategy.

- More detailed knowledge and systematic testing of DNA preservation in local Danish burial contexts are necessary in order to provide further information of the site types that are worth targeting. In particular, Danish environments such as bogs and oak coffin graves have been demonstrated to provide poor chances of retrieving DNA.

Such research results would be particularly valuable when larger sample sets are to be tested. This is less problematic in studies investigating only one sample, in which case it is possible to select an exceptional environment and optimize the protocol specifically.

- In order to be able to select the best possible samples for sequencing in the future, it would be important to test, if the qPCR screening assays applied by Wales et al. 2012 on ancient plant material also is valid on animal bones.

- A more precise focus on teeth material is recommendable. Teeth sampled from the jawbone are generally assumed to be a superior material to bone with regard to the preservation of endogenous DNA. Furthermore, their composition makes them less prone to contamination. Therefore, teeth should be a targeted test material in the future.

2) Method improvement.

- The performance of target capture sequencing was most likely challenged by the low input of endogenous DNA of the DNA libraries in the experiments conducted in the present study. The amount of endogenous DNA, however, could potentially be increased by new optimized aDNA extractions and a new method for ssDNA library preparation that retains small, damaged DNA fragments that would have been lost in dsDNA library preparation. If ssDNA libraries were to be used for capture, it would, however, be preferable if the capture were optimized to also retain smaller fragments.
- Wool is a complex and thus challenging genetic trait to study as it is encoded by many genes. Less complicated characteristics related to sheep wool could be easier to target in future studies. Such traits could include wool colour and the gene controlling wool shedding. Even the development of genes for other sheep products, such as meat, could provide relevant information for the divergence of breeds, breeding strategies, and the product targeted.

3) Alternative methods

- Samples with extremely good preservation of DNA, such as the perma-frozen sheep skin from the Iceman Ötzi's clothing, could be analysed with the SNP50 BeadChip, which is able to place modern sheep into breeds (Kijas et al., 2009). Although ancient sheep are not expected to be genetically identical to any modern sheep population, the analysis could provide evidence for its kinship with, for instance Nordic short-tailed sheep breeds.
- The research by Caroline Solazzo on ancient keratins demonstrates the potential to study the development of ancient keratin-associated proteins (KAPs) (Solazzo et al., 2013), which I also investigated in this PhD thesis by instead using DNA. Solazzo is now aiming to study keratins in archaeological materials. Based on my experiences and results in chapter 2 of this thesis, the analysis of keratins provides a promising future path, even for the ancient Danish textiles from the degrading environments of the oak coffins and the peat bogs.

4) Promising materials

- If obstacles with extracting DNA from sheep bones can be overcome, sheep bones or teeth from the previously mentioned tell site Százhalombatta-Földvár could represent a unique case study for the development of sheep wool. At Százhalombatta-Földvár, sheep bones from before and after 2000 BC display two different kill-off patterns, of which the latter is typical for flocks being kept for their wool. It would, therefore, be important to examine sheep bones from before and after 2000 BC, for thus to sample and compare them genetically. This will potentially allow for suggesting new hypotheses

about the changing characteristics of domesticated sheep, especially concerning their capacity to produce wool.

- When textiles are sampled for DNA in the future, they should be selected from very specific environments. The textile from Krogens Mølle Mose, which was previously shown to contain amplifiable DNA (Brandt et al., 2011) could, for instance, be analysed with the capture approach applied here, in order to target its complete mitochondrial genome and to test and assess if nuclear DNA can be obtained. This sample would, moreover, be a significant specimen, as strontium isotope analysis has already been performed on it, indicating that the wool from which it was produced was non-local (Frei et al., 2009b).

Other conclusive factors that should be considered:

1) The results of strontium isotope analyses have demonstrated the presence of non-local textile materials in Danish prehistoric societies. This is not surprising based on the large amounts of other imported artefacts. However, it is only recently that the value and importance of textiles in ancient societies have been explored and highlighted since these were previously mostly considered local and of little economic value..The geochemical analyses have furthermore demonstrated the possibility that textiles found in Denmark are not necessarily local. This has significant ramifications for the archaeological interpretation of genetic analyses and should be kept in mind when studying wool development, as the studied objects may indeed not reflect wool development locally in Denmark.

2) Different types of sheep or breeds were present in ancient times, and they may have been used for different purposes such as meat production or wool production. Therefore, when studying sheep wool development this needs to be kept in mind. A sound strategy is to select samples from textiles, as these provide information about those sheep that were used for wool production. This is, however, not possible for all types of analysis. Therefore, the context of bone samples and its evidence for the various uses of sheep becomes crucial.

3) I am convinced that the strongest and most solid results will derive from the combination of several of the methods outlined above. Strontium isotope analysis, the analysis of ancient DNA, and conventional fibre analysis provide very different types of information, which are all crucial to the investigation of the development of sheep wool. Therefore, the methodological sound combination of these analyses is the optimal solution to continue the investigation of wool development in the future.

The second, third and fourth chapters in part II dealt with the species identification of skins. The studies yielded the following conclusions:

1) The analyzed skins from the Danish Iron Age capes all derive from domesticated animals. The dominance of domestic animals corresponds to the pattern seen in examined bone assemblages from contemporary Pre Roman and Early Roman Iron Age settlement sites. This confirms that wild animals play only a minor role as game in the Iron Age, and that the economy and clothing relied primarily on domestic animals (Kveiborg 2008: 61).

2) For samples not deriving from highly degrading environments, traditional PCR is still a valuable tool for species identification, although short sequences may not allow further resolution than the species level. In such cases, NGS approaches, such as capture could be attempted to retrieve the complete mitochondrial genome. However, PCR is a cheaper, and a more common technique, and thus more available for most archaeological projects.

3) Even samples from presumably optimal environments for DNA preservation do not always provide amplifiable DNA. In such cases, and for samples from degrading environments, species identification can be attempted by alternative methods, such as PMF or MS-based peptide sequencing.

4) The second study, in which mass spectrometry-based peptide sequencing was applied to skins from the Danish peat bogs, demonstrated that MS-based peptide sequencing is a reliable method for species identification with several advantages:

- First, MS-based peptide sequencing is applicable to materials from highly degrading environments, as for instance the Danish peat bogs, from which DNA has now been proven impossible to extract. This gained knowledge and the applied method are valuable for the large material of skins from the Danish peat bogs and other degrading burial environments in general.
- Second, the analysis yielded clear peptide identifications that make the species identifications by mass spectrometry highly reliable.
- Third, mass spectrometry yielded several additional and novel results, including the finding of bovine foetal haemoglobin in one skin sample, indicating that the skin came from an animal slaughtered within the first month of its birth. This demonstrates that MS-based peptide sequencing has the potential to yield unique information, which is not obtainable via the analysis of ancient DNA. Furthermore, this adds to the scholarly discussions among archaeologists and anthropologists concerning the choice of skins selected for garments in the past.

5) The species identification of skins from the Danish bogs demonstrated that microscopy species identification of archaeological samples holds two particular challenges: often only modern comparison material is available, and the state of archaeological hairs is mostly degraded.

6) Online public databases of protein sequences are not complete at present. They do, however, already enable the identification of the most common domesticated species. Nevertheless, future

expansions of the databases will make the identification of more proteins and species possible. This could, for instance provide the potential to species identify the Saqqaq skin samples of the fourth study, which at the moment are not be possible to identify further than to the level of family by PMF analyses.

7) Although PMF-based approaches allow relatively fast and inexpensive characterization, making this approach ideal for large-scale applications, the maximization of molecular recovery and data interpretation are crucial when applying even minimally destructive analyses to irreplaceable material of high cultural heritage value. Despite the necessity to sacrifice small parts of archaeological objects in the process, the collection and public sharing of the richest possible set of molecular information, compatible with technology and knowledge available at the time of analysis, is of infinite value for the understanding of our prehistory.

Recommendations: future analysis of species identification of skins should focus on:

1) Producing an atlas of archaeological hair samples verified by MS-based peptide sequencing would provide a more secure comparison material for microscopic analysis of archaeological animal hairs. This would be highly valuable, as microscopy is faster, more accessible and cheaper to apply to a larger material than protein sequencing, which is beyond the reach of most archaeological research budgets.

2) Species identification of all skin elements in one Danish Iron Age cape with MS-based peptide sequencing would be ideal in order to confirm, verify and consolidate results from microscopy stating that the capes are made of skins from the same animal species. It would be important to determine if skins from one or several species are included in a single cape, and if several, which elements they were chosen for. This would provide information on the strategies for the selection of a skin for specific elements based on its properties, as for instance its size, thickness, and flexibility.

Concluding remarks

Overall, the success of the analysis of ancient biomolecules is closely connected to the nature and preservation conditions of the sample; furthermore, the potentials or ability to answer archaeological questions depends on the questions asked and the methods chosen.

As stated at the very outset of the introduction, the use of natural scientific methods has a long history in Danish archaeology (Gram 1891: 97).

Archaeological sciences have their strength in their ability to contribute to our knowledge by addressing issues that cannot be investigated by traditional archaeological methods. While I do not believe that natural scientific results can be included in archaeological analyses as objective facts, as they are formed by the ruling paradigms and the techniques and theoretical background available at a certain time, I have in this study demonstrated that the accurately defined practices and reproducibility of scientific methods hold the potential to provide characterizations of archaeological material properties. These properties and qualities have an impact on human society which can be explored further. Material properties of skin and textiles and the characterization of raw materials are amongst the issues that are difficult to investigate archaeologically. To further investigate the interwoven relationships between textiles, society and raw materials, new approaches to study and characterize these are needed, including developing and testing new methods.

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Appendix.

Co-author statements

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Afhandlingens titel:

Species identification of skins and development of sheep wool

An interdisciplinary study combining archaeology and biomolecular methods

Denne medforfattererklæring gælder for følgende artikel:

Species Identification of Archaeological Skin Objects from Danish Bogs: Comparison between Mass Spectrometry-Based Peptide Sequencing and Microscopy-Based Methods

Den ph.d.-studerendes bidrag til artiklen

Contributed to conceiving and designing the experiments
Performed the experiments
Contributed to data analysis
Wrote main parts of the manuscript and managed the manuscript.

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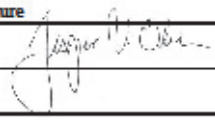
31.10.2014	Jesper V. Olsen	

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Dear Luise

The given information to the attached co-author statement is correct.

Med venlig hilsen / Best regards

Anne Lisbeth Schmidt
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Re: Co-author statements for PhD thesis

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Dear Luise

The given information to the attached co-author statement is correct.

Let me know if there is anything else to do.

have a nice evening and week end

best,

Mathilde Sarret

Le Mercredi 29 Octobre 2014 15:41 CET, Luise Ørsted Brandt <luisebrandt@hotmail.com> a écrit:

> > > Dear all,
> > Thank you again for the collaboration on the species identification article. I think it is a very nice study.
> > I am about to hand in my PhD, in which the article is ment to be included. I therefore hope you will agree to the attached co-author statement.
> As I realize that all of you may not have digital signatures, I have been allowed to include either scanned signatures or emails stating that the given information is correct.
> > Best regards,
> Luise
>

Supporting information for:

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Species identification of archaeological skin objects from Danish bogs: comparison between mass spectrometry-based peptide sequencing and microscopy-based methods

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Text S1. Ancient peptide sequencing and identification: methods

Mass Spectrometry-based peptide sequencing sample preparation, procedure A

For each ancient skin sample, two subsamples of 11-48mg were cut into small pieces with a scalpel. Each subsample was then transferred in a protein LoBind 1.5mL tube (Eppendorf, Germany). Each of the two subsamples was processed independently as follows. Each sample was re-suspended in 500µL of 100mM ammonium bicarbonate solution at pH 8.00, it was shaken vigorously for 1min and it was pelleted by centrifugation at 14000g for 5mins. The supernatant was discarded. This step was performed twice. The pellet was re-suspended in 1mL 1.2M HCl and incubated at 4˚C for 24h. It was then pelleted by centrifugation at 14000g for 10mins. The supernatant was collected and dried in a centrifugal evaporator for 20mins at room temperature. The pellet generated after evaporation was re-suspended in 200µL 50 mM ammonium bicarbonate pH 8.00. The pH was checked using pH-indicator strips and adjusted to 8.00 with concentrated ammonium hydroxide and the solution was incubated at 70˚C for 24h. After centrifugation at 14000g for 10mins, to precipitate any eventual insoluble residue, the supernatant was collected and transferred in a fresh protein LoBind 1.5mL tube. Digestion was then started adding 4µL of 0.5µg/µL sequencing grade trypsin solution (Promega, Nacka Sweden) and incubating at 37˚C overnight. The following morning 2µL of fresh trypsin were added and digestion was extended for 6 additional hours. Digestion was quenched with 10% trifluoroacetic acid to a final concentration of 0.2-0.8% to reach pH < 2.00 and tryptic peptides were immobilised on C18 stage tips, as described in procedure B.

LC-ESI high-resolution MS/MS analysis, procedure A

All peptide mixtures were analysed by online nanoflow reversed-phase C18 liquid chromatography tandem mass spectrometry (LC-MS/MS) as described previously [1].

Mass Spectrometry-based peptide sequencing sample preparation, procedure B

Generation of tryptic peptides from the second group of archaeological bog skin samples was performed using a filter-aided sample preparation (FASP) protocol [2], modified for ancient and degraded samples [3]. For each ancient skin sample a subsample of 8-53mg was cut into small pieces with a scalpel and transferred in a protein LoBind 1.5mL tube (Eppendorf, Germany). The sample was suspended in a 300µL lysis buffer (4% SDS, 0.1M DTT, 0.1M Tris/HCl, pH 8.00), manually homogenised using a sterile micropestle (Eppendorf, Germany) and thereafter heated to 95°C under agitation for 10mins. The samples were then centrifuged at 14000g for 10mins to precipitate insoluble debris. The supernatant (SDS-fraction) was mixed with 2mL 8M urea in 0.1 M Tris/HCl pH 8.00 and ultrafiltered at 4500g, for the time necessary to remove all the solvent except what remained in the dead volume, through an Amicon Ultra-4 (Merck Millipore, USA) centrifugal filter unit with 10kDa NMWL. Sample preparation continued with washing the fraction retained above ultrafilter with 2mL 8M urea in 0.1M Tris/HCl pH 8.00. Alkylation was achieved by re-suspending the fraction retained above ultrafilter in 500µL of 50mM 2-Chloroacetamide (CAA), 8M urea 0.1M Tris/HCl pH 8,00 and incubating for 20mins in the dark at room temperature. CAA was removed by washing with 1mL of 8M urea 0.1M Tris/HCl pH 8.00 twice, finally urea was removed by washing twice with 1mL 50mM ammonium bicarbonate, pH 7.50-8.00. The fraction retained above ultrafilter was re-suspended in 300µL of 50mM ammonium bicarbonate pH 7.50-8.00 and mixed. One microliter of solution was collected for protein quantification using a Qubit fluorometer (Invitrogen-Life Technologies, USA).

Protein digestion was started by adding 4µL of 0.5µg/µL sequencing grade trypsin solution. After mixing, pH was checked using pH strips and, when necessary, adjusted to

7.50-8.00. The ultrafiltration units were transferred into new 15mL tubes, sealed with parafilm and incubated overnight at 37˚C. The following morning two supplemental microliters of fresh trypsin solution 0.5µg/µL were added and digestion was extended for an additional 6 hours. Ultrafiltration units were then centrifuged at 4500g for 10mins to collect the digested peptides and 1µL of the filtrate was collected for Qubit protein quantification. An additional 500µL aliquot of 50mM ammonium bicarbonate pH 7.50-8.00 was added to the filter, mixed, and centrifuged at 4500g for 10mins to elute possible remaining peptides. The filtrate was then transferred to a 1.5mL protein LoBind tube and acidified with 10% trifluoroacetic acid to a final concentration of 0.2-0.8% to reach pH < 2.00. C-18 solid phase extraction (SPE) Stage tips were prepared in-house and sequentially conditioned with 150µL methanol, then 150µL 80% acetonitrile solution (80% acetonitrile, 0.5% acetic acid, 19.5% ddH_2O -v/v/v-), and finally 150µL 0.5% acetic acid in ddH_2O (v/v) [1,4] The acidified peptides were then loaded into the stage-tips and immobilized onto the C-18 filter by centrifugation. Next, the filter was washed with 150µL 0.5% acetic acid in ddH_2O, centrifuged until dry, and stored at -20˚C. Immediately before LC-MS/MS analysis, tryptic peptides were eluted from the Stage-tips membrane, using 10µL of three solutions of progressively more concentrated acetonitrile (40-60-80%, 0.5% acetic acid, in ddH_2O -v/v/v-), directly into a 96-well plate. The samples in the plate were concentrated in a centrifugal evaporator to a volume of approx. 2-4µL and re-suspended in 1% TFA to reach a final volume of 10µL.

NanoLC-ESI high-resolution MS/MS analysis, procedure B

The LC-MS system consisted of an EASY-nLC system (Thermo Scientific, Odense, Denmark) interfaced to the Q Exactive (Thermo Scientific, Bremen, Germany) through a nano electrospray ion source. For each peptide sample 5µL were auto-sampled onto and directly separated in a 15cm analytical column (75µm inner diameter) in-house packed

with 3µm C18 beads (Reprosil-AQ Pur, Dr. Maisch) with a 65mins linear gradient from 5% to 26% acetonitrile followed by a steeper linear 14min gradient from 26% to 48% acetonitrile. Throughout the gradients a fixed concentration of 0.5% acetic acid and a flow rate of 250nL/min were set. A final washout and column re-equilibration added an additional 11mins to each acquisition. The effluent from the HPLC was directly electrosprayed into the mass spectrometer by applying 2.0kV through a platinum-based liquid-junction. The Q Exactive was operated in data-dependent mode to automatically switch between full scan MS and MS/MS acquisition. Software control was Tune version 2.0-1428 and Excalibur version 2.2.42. The settings used were as described for the "sensitive" acquisition described by Kelstrup et *al*. [5]. Each full scan MS was followed by up to 10 MS/MS events. The isolation window was set at 2Th and a dynamic exclusion of 90 seconds was used to avoid repeated sequencing. Only precursor charge states above 1 and below 7 were considered for fragmentation. A minimum intensity threshold for triggering fragment MS/MS was set at 1e5. Full scan MS were recorded at a resolution of 70,000 at m/z 200 in a mass range of 300-1700 m/z with a target value of 1e6 and a maximum injection time of 20 ms. Fragment MS/MS were recorded with a fixed ion injection time set to 120 ms through a target value set to 1e6 and recorded at a resolution of 35,000 with a fixed first mass set to 100 m/z.

Protein identification of spectra from procedures A and B

Raw files generated during spectra acquisition were searched on a workstation using the MaxQuant algorithm v. 1.2.2.5 [6] and the Andromeda peptide search engine [7] initially against a target/reverse custom-made list of all the mammalian Alpha-1 type I, Alpha-2 type I and Alpha-1 type III collagen sequences publicly available in UniProt and nrNCBI (approximately 240 accessions in total), and then against the target/reverse protein list in

the *Bos taurus* and *Homo sapiens* reference proteomes (34365 and 84946 -including isoforms- accessions downloaded from UniProtKB on Oct. 24 2012 and Dec. 31 2012, for *B. taurus* and *H. sapiens* respectively) together with the complete list of proteins available in NCBI RefSeq after taxonomical restriction to *Ovis aries* (22752 accessions, downloaded on Jan. 8, 2013) and *Capra hircus* (31461 accessions, downloaded on Dec. 2, 2013). In every search, spectra were also matched against the common contaminants, such as wool keratin and porcine trypsin sequences, downloaded from Uniprot.

Trypsin was selected as the proteolytic enzyme and two missed cleavages were allowed. Oxidation (M and P), deamidation (N and Q), acetylation (K), G→pyro-Glu (N-term Q), and E→pyro-Glu (N-term E) were selected as variable amino-acid modifications. Carbamidomethylation (C) was selected as fixed modification. Default values were used for precursor (6 ppm) and fragment (20 ppm) ions mass tolerance. False-discovery rate was set at 1% and minimum peptide-score and peptide sequence length were set at 80 and 6 respectively. The amount of random matches was evaluated performing MaxQuant search against reversed sequences. For the identification of species-diagnostic peptides, possible environmental contaminants, such as actin, tubulin, keratins and keratin-associated proteins, as well as proteins commonly used in mass spectrometry facilities as standards or calibrants and proteins highly conserved, such as histones, and all human proteins were excluded from further investigation. A minimum of two unique peptides was required for confidently calling proteins. Using locally installed, command line operated, Blastp algorithm, part of the stand-alone BLAST suite "BLAST+" [8], protein-unique peptides from this subset of identified proteins were remotely aligned, `-remote`, against the entire nrNCBI protein database, `-db nr`, using optimization for short

sequences, `-task blastp-short`, allowing no gaps, `-ungapped`, and not applying composition-based statistics, `-comp_based_stats F`. Those peptides presenting 100% alignment, or alternatively a fragmentation pattern exclusively compatible with either *Ovis, Capra* or *Bos* genus among mammals (after excluding any possible I/L isobaric substitution, but contemplating possible deamidation events), and excluding any possible origin from biological contaminants in soil (i.e. bacteria and fungi), were reported as species-diagnostic for either *Ovis aries, Capra hircus* or *Bos taurus*. We also identified "semi-specific" peptides not exclusively matching sheep/goat or cattle, when the nature of the sample excluded any other plausible alternative assignment.

Supplementary figures

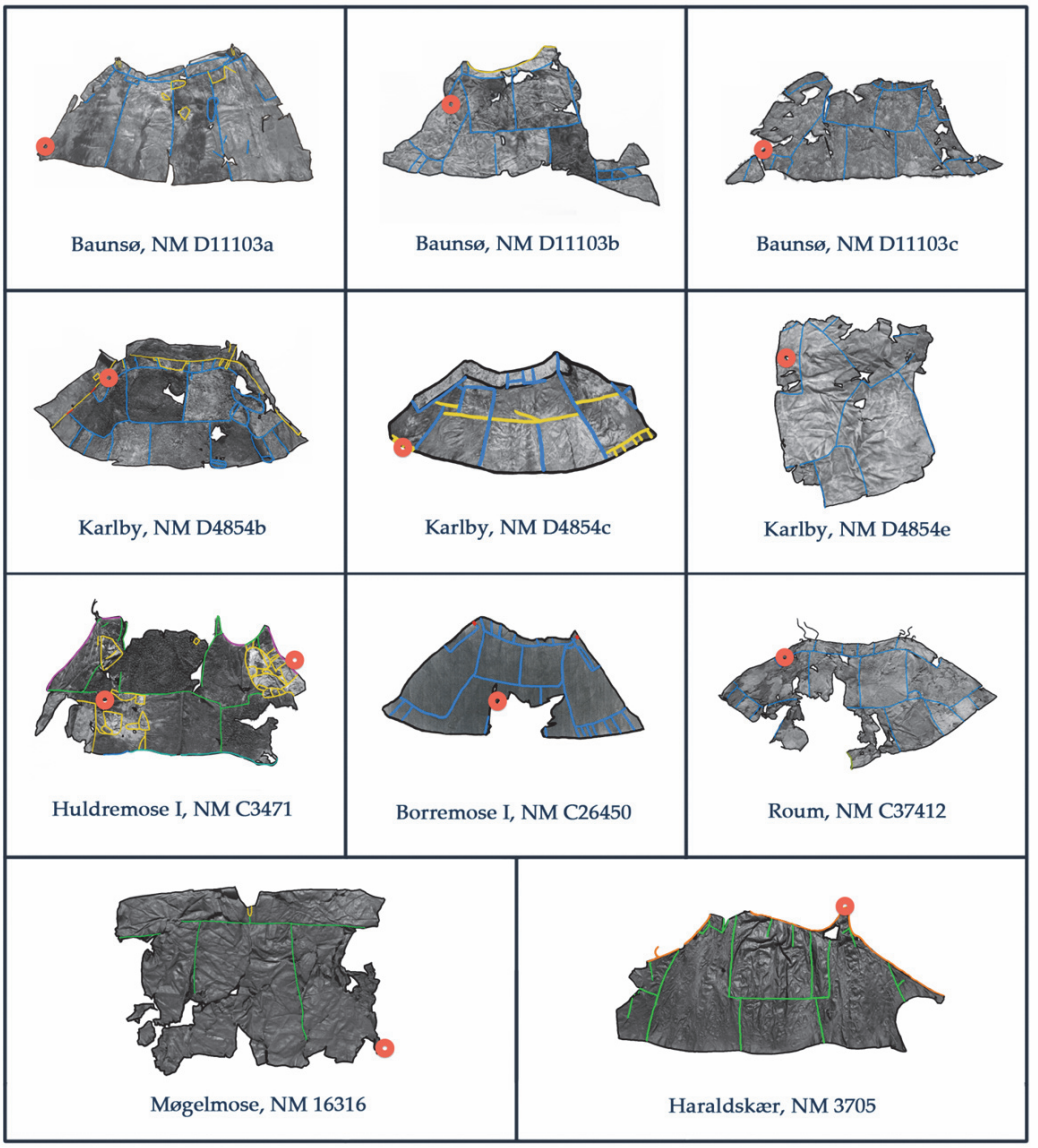

Figure S1. The archaeological skin objects investigated. Coloured lines indicate the seams joining skin elements composing each garment. Red rings indicate the sampling points for all three analyses. For the Huldremose I cape, two elements were sampled. The sample referred to as "dark" is the one on the right. Photos by Roberto Fortuna, the National Museum of Denmark.

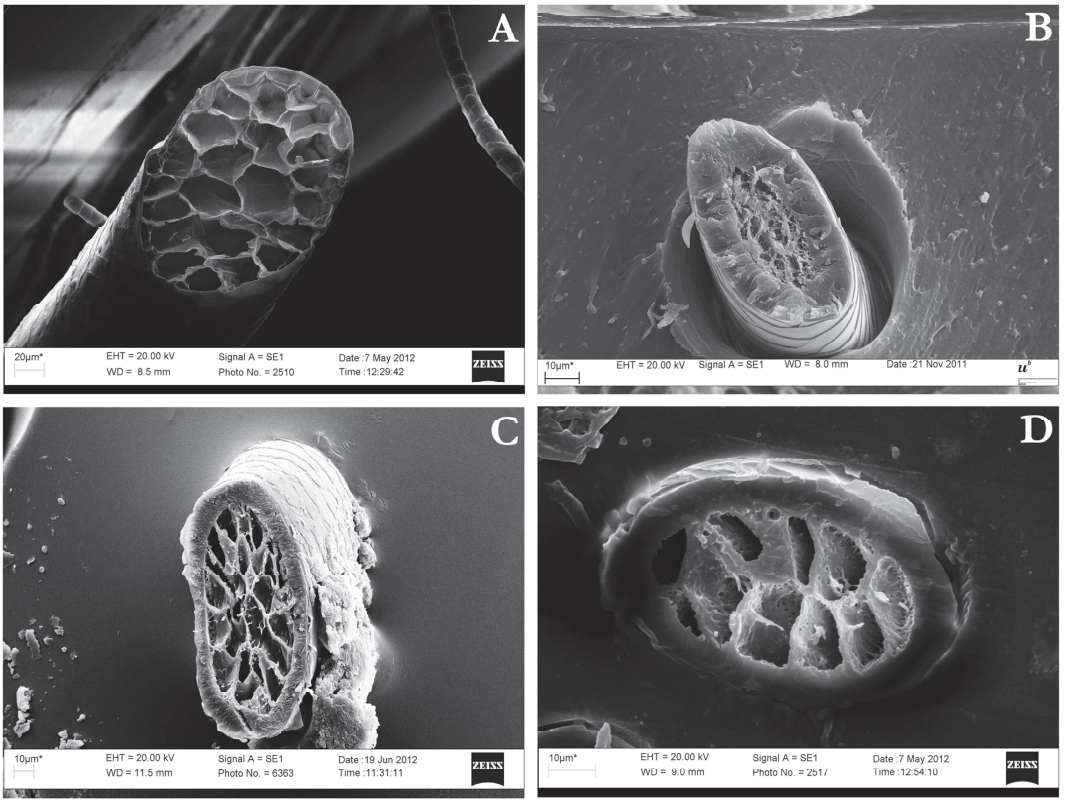

Figure S2. SEM pictures of modern cross-sectioned hairs of the most common domesticated species used as reference material. (A) sheep, (B) cattle, (C) goat, (D) horse.

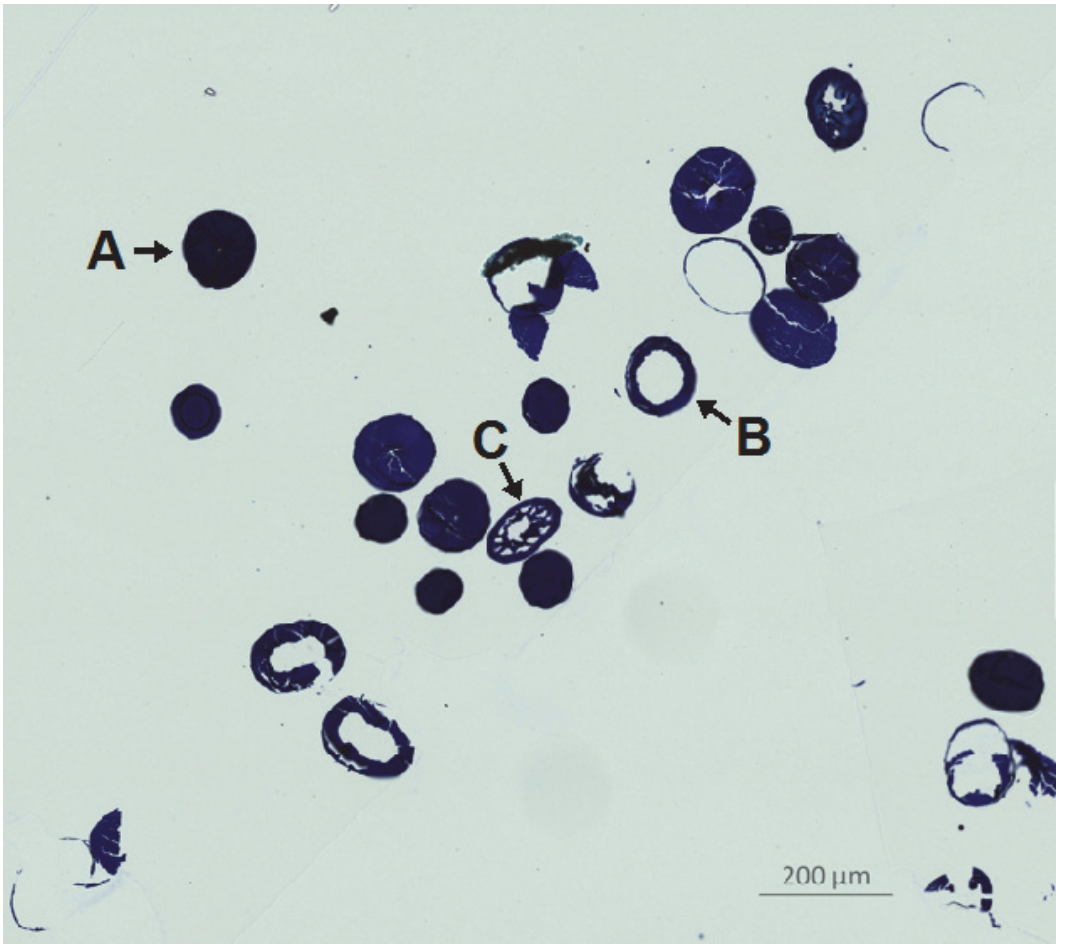

Figure S3. Example of evidence used for species identification by transmitted light microscopy. Microscopy of the cross-section of hairs from sample 1 shows three different variants of guard hair: (A) round hair with continuous medulla, approximately 80-130 µm wide. The medulla displays wavy lines and an unfilled center without pigmentation. The cuticular outer layer poorly preserved; (B) round to oval hair with absent medulla, approximately 100 µm wide; cortex approximately 30 µm thick. The cuticular outer layer poorly preserved; (C) round to oval hair with open, wide lattice medulla, approximately 100 µm wide. The cuticular outer layer poorly preserved. The size and shape of hair as well as the absence of medulla in some hairs indicated cattle, when compared to modern cattle hair [9-16] and present-day contemporary samples.

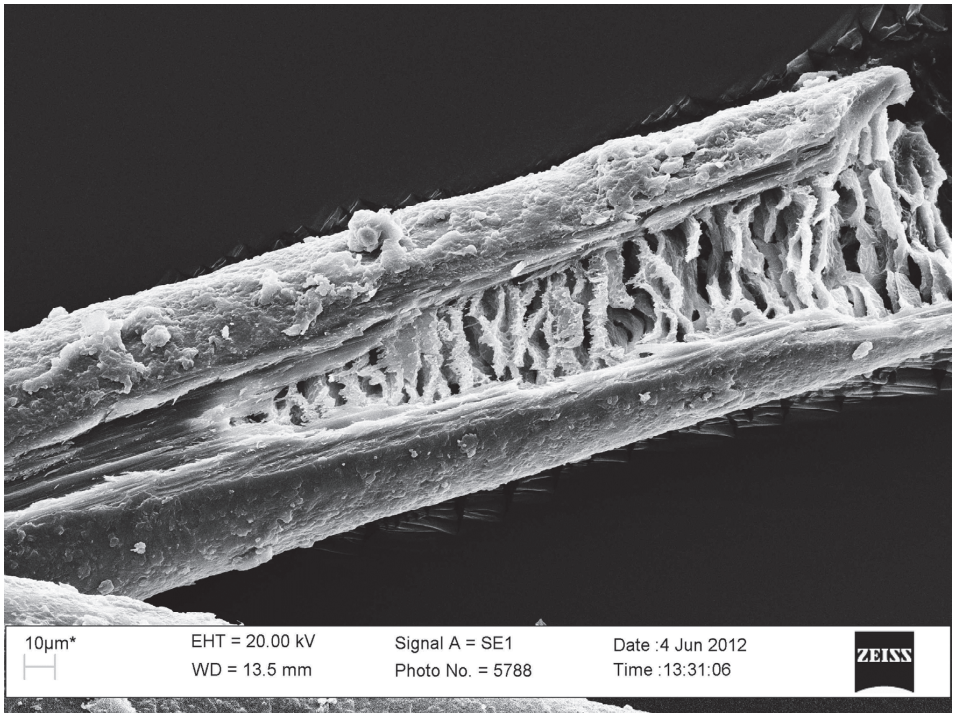

Figure S4. SEM of hair for species identification by Method 2. For sample 1, scales are poorly preserved or missing. When the medulla is preserved, the cross-section shows fields with a dot-like structure. Compared to the sample displayed, modern sheep have a much finer epidermis and do not display the dot-like structure of medulla chambers. Cattle have very fine "bubbles" filling in the medulla, which appear very different from the sample. Goats and horses have a similar epidermis size and medulla, including a dot-like structure in the medulla chambers. Goats and horses can be difficult to distinguish, especially if one or more of the three criteria looked for are poorly preserved. The sample was designated as horse skin by light microscopy, as the fine lines at the edge of the medulla before the epidermis, specific for goats, were not visible.

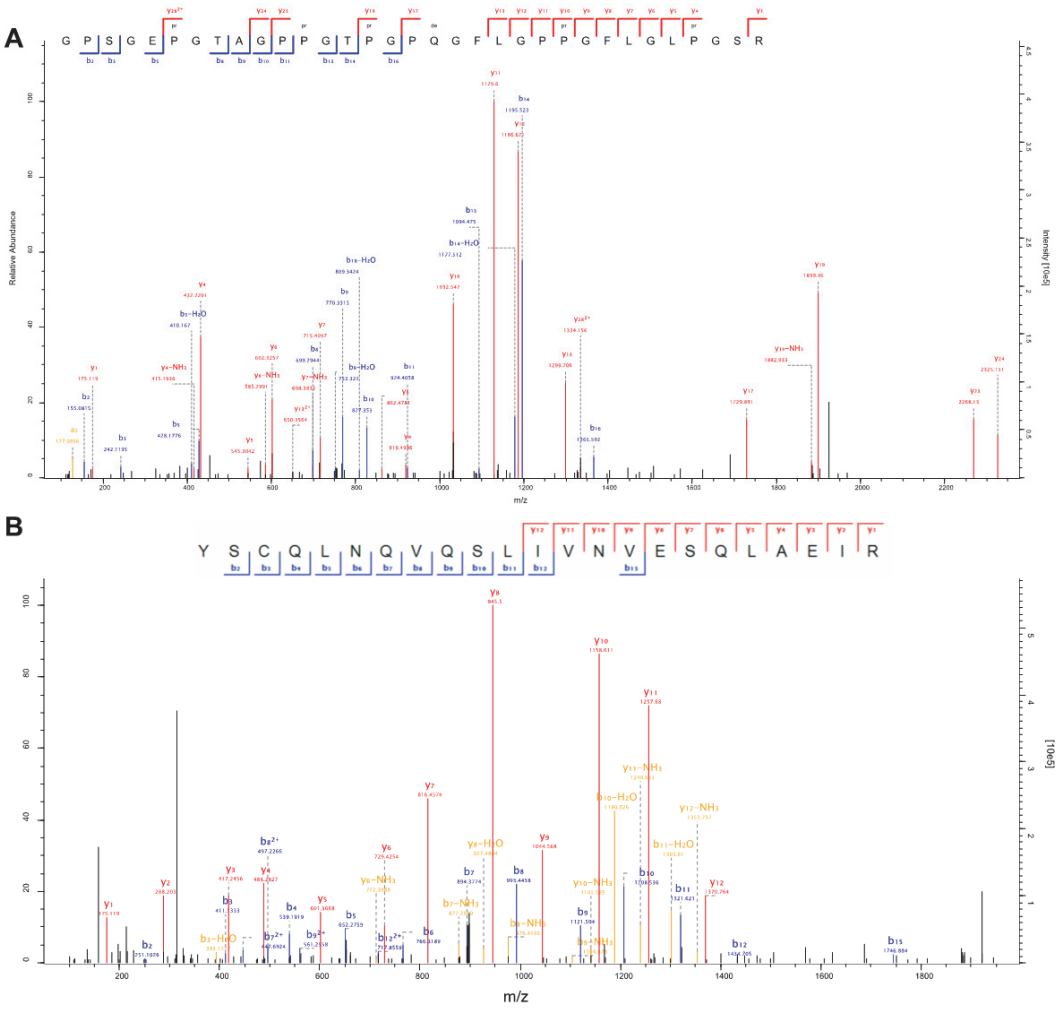

Figure S5. Examples of tandem spectra supporting the identification of sample 1 as goatskin. (A) Type I alpha-2 collagen (COL1A2) diagnostic peptide in the goat-diagnostic version [17,18] and (B) peptide belonging to keratin33A previously assigned to goat [19].

Supplementary Tables

Fibre	Scales of primary hairs	Cross section	Medulla
SHEEP fine 10-20um, large up to 150um	mosaic, straight margins	round-oval fine cortex	cloisonné
GOAT fine 10-15um, large up to 200um	rippled, fine horizontal, intervals, 5-10um	oval or kidney-shape SEM: cloisonné	SEM: cloisonné LM: flatten disk margins fringed often large and dark
HORSE fine 10-20um, large up to 120um	fine straight or rippled, intervals 10um	oval LM: medulla „star“ SEM: cloisonné	LM: disk-fields SEM: cloisonné margins flat mane: irregular, small
CATTLE fine: 20 um, large	straight-wave, wide intervals, 10-15um	round-oval SEM: cloisonné	granulated (LM/SEM) irregular width

Table S1. Morphological traits used for species identification of the commonest domesticates by Method 2 (light microscopy and SEM). The “X” symbol, in red, indicates cases in which a morphological trait differs sufficiently to distinguish between two species. The “Z” symbol, in blue, indicates cases where two species share a morphological trait. The table illustrates how species are identified based on morphological characteristics of the medulla, cross-section and scales, and demonstrates where the species differ and where similarities cause problems in species identification. For example, sheep and goat can only be differentiated by the appearance of the primary follicles as the fine fibres are too similar. Evaluation of scale height, which is used in today’s industry to differentiate sheep wool from cashmere, is only possible in extremely well preserved archaeological samples. Distinguishing the medulla of sheep and goat hairs is difficult as it is often either degraded or the fibres are too darkly stained to make them visible under light microscopy. On the other hand, the table shows that cattle can be distinguished from the other species by their granulated medulla, which is visible on cross-sectioned fibres using light microscopy or in SEM if the fibres are not too dark.

Sample no.	Sample	Recovery (mg pt/ g sample)	Total ident. prot.	Total ident. pept.*	Total ident. MS/MS spectra*	Prot. after filtering	Unique pept. after prot. filtering	MS/MS spectra after prot. filtering**
1	Baunsø NM D11103a		110	655 (367| 521)	31730 (16513+15217)	18	55	213
2	Baunsø NM D11103b	1.32	29	144	32029	2	33	56
3	Baunsø NM D11103c	2.59	42	201	30497	5	26	41
4	Borremose I NM C26450		108	739 (400|605)	37770 (20491+17279)	18	81	437
5	Huldremose I NM C3471 dark		119	841 (477|662)	42828 (21246+21582)	23	140	932
6	Huldremose I NM C3471 light		132	772 (379|603)	44570 (21940+22630)	26	65	240
7	Karlby NM D4854b	20.13	107	528	33796	18	81	225
8	Karlby NM D4854c	7.54	77	368	34781	14	46	110
9	Karlby NM D4854e	10.00	55	243	27168	5	25	53
10	Møgelmose NM 16316		139	739 (373|559)	38656 (16179+22477)	23	66	194
11	Roum NM C37412	8.52	13	81	21541	1	12	17
12	Haraldskær C3705		108	678 (321|583)	38108 (19161+18947)	23	78	369
	Modern sheep		362	1078	11728	127	437	3679
	Modern goat		273	779	9664	66	273	3206
	Modern cattle		177	480	2366	28	123	462

Table S2. General statistics on protein and peptide recoveries for each sample. *Including contaminants. ** Relative to unique peptides. Protein recoveries were measured only for skin samples prepared with procedure B.

Accession number	Protein Name	Unique Peptides	Uniq. Pep. Seq. Cov. [%]	Seq. Length	Matched spectra	
P02081	Hemoglobin fetal subunit beta	3	17.9	201	18	
	Sequence	Length	Mass	Charges	MaxQuant Score	Matched spectra
	AAVTSLFAK	9	906,51747	2	91.318	1
	FGSEFSPELQASFQK	15	1700,8046	2	102.73	1

Table S3. Peptides supporting the identification of Hemoglobin fetal subunit beta in sample 10 (Møgelmose, NM 16316).

Sample	No.	Accession number	Protein Name	All Matching Peptides*	Unique Peptides	Total Seq. Cov. [%]**	Uniq. Pep. Seq. Cov. [%]	Seq. Length	Matched spectra **
Baunsø NM D11103a	1	NP_001272646.1	hair acidic keratin 1	34	1	64.4	5.6	413	394
	2	XP_005680154.1	keratin. type II cuticular Hb1-like	42	5	73.6	10.4	402	382
	3	XP_005693870.1	keratin. type I cuticular Ha4-like	36	4	60.7	8.8	433	456
	4	BAJ65377.1	keratin33A	29	1	59.7	5.7	404	395
Borremose I NM C26450	1	gi|426220723	collagen alpha-1(III)	31	7	29	9.4	1467	170
Haraldskær NM C3705	1	gi|426227338	collagen alpha-2(I)	56	23	50.1	24.6	1364	458
	2	XP_005693869.1	keratin. type I cuticular Ha8-like	12	5	28.8	13.9	476	47
	3	NP_001272646.1	hair acidic keratin 1	35	2	67.1	5.6	413	791
	4	XP_005678993.1	collagen alpha-2(I) chain	60	60	57.3	57.3	1364	445
	5	NP_001272697.1	keratin associated protein 12.1	2	2	19	19	116	2
	6	XP_005680154.1	keratin. type II cuticular Hb1-like	41	5	72.1	10.4	402	801
	7	BAJ65365.1	keratin33A	34	1	72	4.2	404	738
	8	XP_005693870.1	keratin. high-sulphur matrix protein. IIIA3-like	36	4	65.8	10.4	433	874
Huldremose I NM C3471 dark skin	1	gi|426227338	collagen alpha-2(I)	41	29	41.5	31.9	1364	301
	2	gi|426220723	collagen alpha-1(III)	30	5	30.9	6.7	1467	169
Huldremose I NM C3471 light skin	1	gi|426220723	collagen alpha-1(III	43	8	40.8	10.5	1467	218
Møgelsmose NM 16316	1	P02465	collagen alpha-2(I)	51	6	51.2	7	1364	361
	2	Q2KJ32	Selenium-binding protein 1	4	3	10.2	7.8	472	9

Karlby NM D4854b	1	gi|426220723	collagen alpha-1(III)	49	13	47.6	15.1	1467	125
	2	gi|426237753	collagen alpha-1(I)	69	2	58.9	4	1471	338
Karlby NM D4854c	1	gi|426220723	collagen alpha-1(III)	36	9	38.4	11.5	1467	109
Karlby NM D4854e***	1	gi|426227338	collagen alpha-2(I)	28	16	33.1	23.2	1364	55
Roum NM C37412***	1	gi|426227338	collagen alpha-2(I)	15	12	16.9	13.9	1364	21
Baunsø NM D11103b	1	P02465	collagen alpha-2(I)	31	8	34	8.8	1364	64
Baunsø NM D11103c	1	gi|426227338	collagen alpha-2(I)	41	14	41.1	18	1364	77
	2	gi|426237753	collagen alpha-1(I)	55	3	50.1	5.4	1471	142
Modern sheep	1	gi|426230702	perilipin-4	4	4	5.1	5.1	1051	4
	2	gi|426219699	major allergen Equ c 1-like	4	4	18.1	18.1	182	8
	3	gi|426220721	collagen alpha-2(V) chain	24	23	30.5	29.8	1499	47
	4	gi|426259137	serpin A3-1-like. partial	3	2	13.2	9.8	204	4
	5	gi|426241977	uncharacterized protein LOC101116248	2	2	6.7	6.7	564	6
	6	gi|426251176	tenascin-X	13	4	6.4	1.6	3949	16
	7	gi|426258629	complement C3-like	8	2	14.9	4.1	657	13
Modern goats	1	AAX45026.1	immunoglobulin gamma-1 chain F7-299	1	1	3.8	3.8	239	1
	2	XP_005701292.1	odorant-binding protein-like	2	2	17.4	17.4	172	2
	3	XP_005678993.1	collagen alpha-2(I) chain	76	76	70.8	70.8	1364	285
	4	XP_005679869.1	lumican	14	5	37.4	12.6	342	58
	5	XP_005695453.1	alpha-1-antiproteinase-like	7	2	22.3	7.3	381	14
	6	XP_005701928.1	serpin A3-1-like	6	1	29.3	9.1	164	11
	7	ACH86010.1	II alpha globin	3	1	21.8	10.6	142	3

	8	XP_005692313.1	major allergen I polypeptide chain 1-like	1	1	16.1	16.1	93	2
Modern cattle	1	F1MYG5	prelamin-A/C	6	6	11.3	11.3	664	7
	2	F1MJB5	filaggrin	2	2	12.6	12.6	406	2
	3	P02465	collagen alpha-2(I) chain	45	8	48.1	9.1	1364	96
	4	Q28133	allergen Bos d 2	4	3	18	13.4	172	4
	5	E1BB91	collagen. type VI. alpha 3	24	24	10.6	10.6	3170	34

Table S4. Proteins bearing observed species-diagnostic peptides. * Including non-unique peptides. ** Based on all matching peptides. *** For samples 9 (Karlby NM D4854e) and 11 (Roum NM C37412) no single peptide allowed to discriminate sheep from goats. Proteins bearing peptides supporting ovine identification are reported.

Sample	Accession number	Protein name	N.	Sequence	Length (aa)	Mass	Charge	MaxQuant Score	Matched spectra	References.
No 1. Baunsø NM D11103a	NP_001272646.1	hair acidic keratin 1	1	GLLDSEDCKLPCNPCATTNAYGK	23	2583.1404	3	82.475	1	
	XP_005680154.1	keratin. type I cuticular Ha4-like	1	KKYEEEIALR	10	1277.698	3	129.39	1	
			2	LEAAVTQAEQQGEAALNDAKR	21	2212.1084	3	156.67	3	
	XP_005693870.1	keratin. type I cuticular Ha4-like	1	SDLEAQVESLKEELLFLKK	19	2218.2097	3.4	104.84	4	
	BAJ65377.1	keratin33A	1	YSCQLNQVQSLIVNVESQLAEIR	23	2690.3698	3	150.36	6	[19]
No. 2. Baunsø. NM D11103b	P02465	collagen alpha-2(I)	1	GAPGAIGAPGPAGANGDR	18	1504.7383	2.3	132.05	3	
			2	GYPGNAGPVGAAGAPGPQGPVGPVGK	26	2228.1338	2	119.83	1	
			3	HGNRGEPGPAGAVGPAGAVGPR	22	1980.0038	3.4	176.46	3	
			4	IGQPGAVGPAGIR	13	1191.6724	2	113.77	2	Peptide A [17,18]
			5	SGETGASGPPGFVGEK	16	1475.6892	3	111.74	1	
No. 3. Baunsø. NM D11103c	gi|426237753	collagen alpha-1(I)	1	AGEVGPPGPPGPAGEKGAPGADGPAGAPGTPGPQGIAGQR	40	3453.7025	4	167.95	1	
No. 4. Borremose I. NM C26450	gi|426220723	collagen alpha-1(III)	1	GFPGNPGPPGSPGPAGHQGAVGSPGPAGPR	30	2630.2738	3	91.175	1	
No. 5. Huldremose I dark. NM C3471	gi|426227338	collagen alpha-2(I)	1	TGQPGAVGPAGIR	13	1179.636	2.3	110.64	11	Peptide A [17,18]
	gi|426220723	collagen alpha-1(III)	1	GFPGNPGPPGSPGPAGHQGAVGSPGPAGPR	30	2630.2738	3	137.16	1	

No. 6. Huldremose I light. NM C3471	gi|426220723	collagen alpha-1(III)	1	GFPGNPGPPGSPGPAGHQGAVGSPGPAGPR	30	2630.2738	3.4	124	9	
No. 7. Karlby. NM D4854b	gi|426220723	collagen alpha-1(III)	1	GFPGNPGPPGSPGPAGHQGAVGSPGPAGPR*	30	2630.2738	2.3	183.25	7	
	gi|426237753	collagen alpha-1(I)	1	AGEVGPPGPPGPAGEKGAPGADGPAGAPGTPGPQGIAGQR	40	3453.7025	3	211.59	1	
No. 8. Karlby. NM D4854c	gi|426220723	collagen alpha-1(III)	1	GFPGNPGPPGSPGPAGHQGAVGSPGPAGPR*	30	2630.2738	3	125	1	
No. 9. Karlby. NM D4854e**	gi|426227338	collagen alpha-2(I)	1	GEPGPVGAVGPAGAVGPR	18	1543.8107	2.3.4	161.8	5	
			2	GYPGNAGPVGAAGAPGPQGPVGPTGK	26	2230.1131	2.3	121.33	2	
			3	TGEPGAAGPPGFVGEK	16	1469.7151	3	106.42	1	
			4	TGPPGPAGISGPPGPPGPAGKEGLR	25	2220.1651	2	116.48	1	
			5	TGQPGAVGPAGIR	13	1179.636	2.3	118.88	3	Peptide A [17,18]
No. 10. Møgelmose. NM 16316	P02465	collagen alpha-2(I)	1	GAPGAIGAPGPAGANGDR	18	1504.7383	2	125.22	3	
			2	GDIGSPGRDGAR	12	1156.5585	2	134.38	1	
			3	HGNRGEPGPAGAVGPAGAVGPR	22	1980.0038	2.3	200.45	2	
			4	IGQPGAVGPAGIR	13	1191.6724	2.3	106.49	2	Peptide A [17,18]
			5	SGETGASGPPGFVGEK	16	1475.6892	2	92.063	1	
	Q2KJ32	Selenium-binding protein 1	1	VQTLTLQDGLIPLEIR	16	1808.0407	2.3	123.29	6	
No. 11. Roum. NM C37412**	gi|426227338	collagen alpha-2(I)	1	GEPGPVGAVGPAGAVGPR	18	1543.8107	2.3	145.55	3	
			2	GYPGNAGPVGAAGAPGPQGPVGPTGK	26	2230.1131	2	87.511	1	
			3	TGEPGAAGPPGFVGEK	16	1469.7151	3	93.237	1	
			4	TGPPGPAGISGPPGPPGPAGK	21	1764.9159	2	118.66	1	

			5	TGQPGAVGPAGIR	13	1179.636	2	112.02	2	Peptide A [17,18]
No. 12 Haraldskær. NM 3705	gi|426227338	collagen alpha-2(I)	1	TGQPGAVGPAGIR	13	1179.636	2	124.6	8	Peptide A [17,18]
	XP_005693869.1	keratin. type I cuticular Ha8-like	1	FGIELAQMQTLISNVEEQLSEIR	23	2647.3527	3	115.54	3	
			2	LAVEEDLCGLHK	12	1382.6864	3	102.4	1	
	NP_001272646.1	hair acidic keratin	1	GLLDSEDCKLPCNPCATTNAYGK	23	2583.1404	3.4	115.54	2	
			2	LPCNPCATTNAYGK	14	1565.6966	2	90.71	1	
	XP_005678993.1	collagen alpha-2(I) chain	1	GPSGEPGTAGPPGTPGPQGFLGPPGFLGLPGS	33	3012.5094	3	123.31	9	
	NP_001272697.1	keratin associated protein 12.1	1	IVYVIPSCQSSR	12	1407.718	2	80.69	1	
			2	PVLYVPVCYK	10	1236.6577	2	82.287	1	
	XP_005680154.1	keratin. type II cuticular Hb1-like	1	KKYEEEIALR	10	1277.698	2.3	178.54	2	
			2	LEAAVTQAEQQGEAALNDAKR	21	2212.1084	3.4	159.14	3	
	BAJ65365.1	keratin33A	1	QNHEQEVNTLQSQLGDR	17	1994.9406	2.3	221.47	11	
	XP_005693870.1	keratin. high-sulphur matrix protein. IIIA3-like	1	SDLEAQVESLKEELLFLK	18	2090.1147	2.3	165.9	6	
Modern sheep	gi|426230702	perilipin-4	1	DVSSQPEEAAAGEVPATGALSR	22	2141.0237	2	90.464	1	
	gi|426219699	major allergen Equ c 1-like	1	ENIIDLTR	8	972.52401	2	106.36	1	
	gi|426220721	collagen alpha-2(V) chain	1	GETGPPGPIGSQGLPGAVGTDGTPGAK	27	2374.1765	2.3	137.32	2	

	gi|426259137	serpin A3-1-like. partial	1	IFADADLSGITGTR	14	1435.7307	2.3	167.14	2
	gi|426241977	uncharacterized protein LOC101116248	1	ISLPFVNSSVSSLTVPSSNIR	21	2203.1848	3	92.474	1
	gi|426251176	tenascin-X	1	LGPISTEGSTAPLEK	15	1498.7879	2	96.591	1
	gi|426258629	complement C3-like	1	LVAYYTLSNANGQR	14	1568.7947	2	83.617	1
			2	VPINDGNGEAILR	13	1366.7205	2	78.884	1
Modern goat	AAX45026.1	immunoglobulin gamma-1 chain F7-299	1	ALEWLGGIR	9	1013.5658	2	105.52	1
	XP_005701292.1	odorant-binding protein-like	1	GDENTLLTHTVNVDEHGK	18	1977.9392	3	151.41	1
	XP_005678993.1	collagen alpha-2(I) chain	1	GPSGEPGTAGPPGTPGPQGFLGPPGFLGLPGSR	33	3012.5094	2.3	264.36	7
	XP_005679869.1	lumican	1	SLEYLDLSFNQITK	14	1669.8563	2.3	239.98	7
	XP_005695453.1	alpha-1-antiproteinase-like	1	VFSNGADLSGITEEQPLK	18	1903.9527	2.3	178.43	8
	XP_005701928.1	serpin A3-1-like	1	VFTSEADLSGITGVR	15	1550.794	2.3	128.15	2
	ACH86010.1	II alpha globin	1	VGSNAGAYGTEALER	15	1493.711	2	107.84	1
	XP_005692313.1	major allergen I polypeptide chain 1-like	1	YNQNPDVLETADILK	15	1731.8679	2.3	120.92	2
Modern cattle	F1MYG5	prelamin-A/C	1	ASASSGAQVGGSISSGSSASSVTVTR	26	2297.1095	2.3	181.69	2

F1MJB5	filaggrin	1	ESSVSQASDSEGYSGDVGR	19	1915.8032	2	187.56	1	
P02465	collagen alpha-2(I) chain	1	GAPGAIGAPGPAGANGDR	18	1504.7383	2	96.993	1	
		2	GEPGPAGAVGPAGAVGPR	18	1515.7794	2.3	151.18	3	
		3	HGNRGEPGPAGAVGPAGAVGPR	22	1980.0038	4	175.32	2	
		4	IGQPGAVGPAGIR	13	1191.6724	2	160.88	2	Peptide A [17,18]
		5	SGETGASGPPGFVGEK	16	1475.6892	2.3	190.24	3	
Q28133	allergen Bos d 2	1	GTSFTPEELEK	11	1236.5874	2	82.261	1	
E1BB91	collagen. type VI. alpha 3	1	LQASVTPLTTPVVSSK	16	1626.9192	2	116.96	1	
		2	QASMDNVK	8	891.41202	2	138.78	2	

Table S5. Species-diagnostic peptides observed. *Peptides matching non-uniquely with the assigned species in the nrNCBI protein database, but incompatible with any other assignments due to the nature of the sample. ** For samples 9 (Karlby NM D4854e) and 11 (Roum NM C37412) no single peptide allowed to discriminate sheep from goats. Peptides supporting ovine identification are reported. References reporting previously identified peptides are cited.

Sample No.	Sample	Peptide version	Mass	Charges	MaxQuant Score	Matched spectra
1	Baunsø NM D11103a	Goat	3012,5094	3	139.67	3
4	Borremose I NM C26450	Sheep	2952,5094	3,4	148.72	14
5	Huldremose I NM C3471 dark	Sheep	2952,5094	3	142.28	6
6	Huldremose I NM C3471 light	Sheep	2952,5094	2,3,4	123.35	10
7	Karlby NM D4854b	Sheep	2952,5094	2,3,4,5	440.92	16
8	Karlby NM D4854c	Goat	3012,5094	2,3,4,5	283.37	6
9	Karlby NM D4854e	Sheep	2952,5094	3,4	156.27	2
12	Haraldskær NM C3705	Goat	3012,5094	3	167.78	11

Table S6. Statistics supporting the identification of the sheep/goat diagnostic collagen type-1 alpha-2 peptide [17,18].

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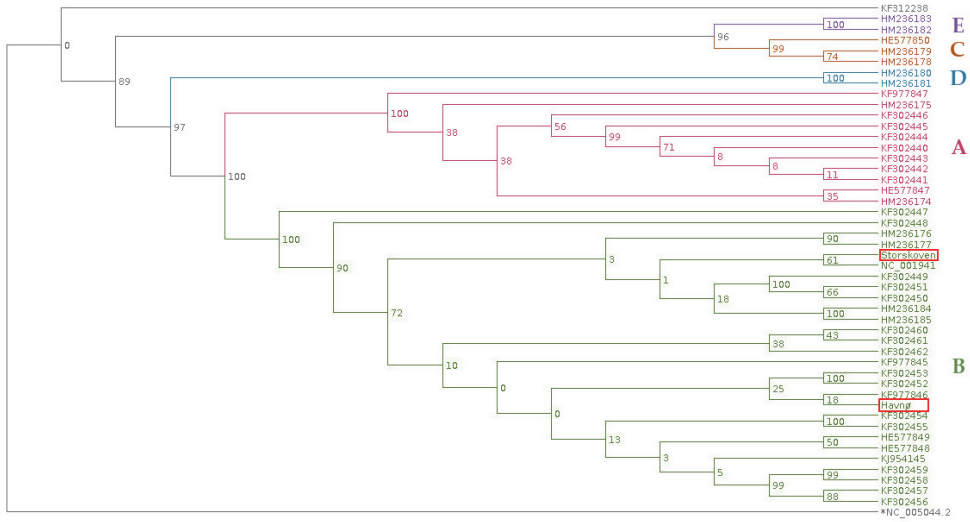

Figure S1. Cladogram of modern sheep based on 45 modern, two ancient complete mitocondrial sequences, and an outgroup, *Capra hircus*. (Large size).

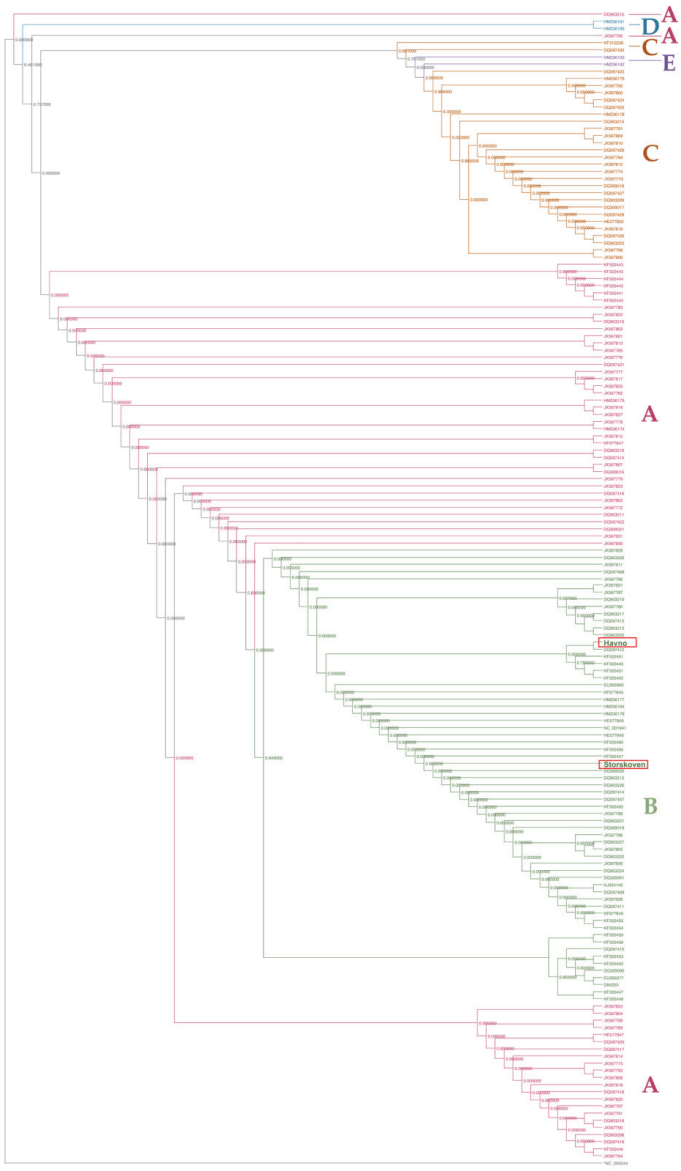

Figure S2. Cladogram of modern sheep based on 159 modern, two ancient complete cytochrome b sequences, and an outgroup, *Capra hircus*. (Large size).

Supplementary tables

Missing positions, bp	
Havnø	Storskoven
1	
389-429	
724-735	
827-853	
945-946	
1031-1062	
1149-1185	
1715-1765	
1815-1852	
2626-2634	
2809-2814	
3205-3215	
3714-3761	
3931-3941	
4116-4139	
4517-4525	
4693-4722	
4950-4959	
5532-5535	
5824-5833	
6140-6147	
6700-6733	

(continued)	
Havnø	Storskoven
6969-6970	
7136-7161	
7798-7839	
7951-7956	
8124-8168	
8364-8376	
9417-9423	
10383-10407	
10765-10776	
11156-11608	
11753-11757	
12357-12367	
13294-13300	
13769-13772	
13905-13916	
15073-15074	
15231-15240	
15659-15909	
	15753-15822
16526-16544	
16577-16618	

Table S1. The uncovered regions of the two almost complete mitogenomes from two ancient sheep bone samples.

Full mitogenome	**Haplogroup**	**Continent**	**Country**	**Breed**
KJ954145	B	No info	No info	Texel
HM236180	D	Middle East	Turkey	Morkaraman
HM236176	B	Middle East	Turkey	Karakas
HM236184	B	Europe	Germany	Muflon
HM236177	B	Middle East	Turkey	Karakas
HM236181	D	Middle East	Turkey	Morkaraman
HM236185	B	Europe	Germany	Muflon
NC_001941	B	Europe	Germany	Merinolandschaf
HM236174	A	Europe	Germany	Merino

Full mitogenome	Haplogroup	Continent	Country	Breed
KF302459	B	Europe	Italy	Merinizzata Italiana
KF302460	B	Europe	France	Lacaune
KF977846	B	No info	No info	Sahelian
KF302448	B	Europe	Italy	Gentile di Puglia
KF977847	B	No info	No info	small-tailed Han
KF302461	B	Europe	Italy	comisana
KF302447	B	Europe	France	Lacaune
KF977845	B	No info	No info	Djallonke
HM236175	A	Europe	England	Romney
KF302454	B	Europe	Italy	Sopravissana
KF302462	B	Europe	Italy	Comisana
KF302453	B	Europe	France	Lacaune
KF302457	B	Europe	Italy	Gentile di Puglia
KF302455	B	Europe	Italy	Gentile di Puglia
KF302452	B	Europe	Italy	Comisana
HE577849	B	Middle East	Israel	Afec-Assaf
KF302456	B	Europe	Italy	Sopravissana
KF302458	B	Europe	Italy	Sopravissana
HE577848	B	Middle East	Israel	Afec-Assaf
KF302442	A	Europe	Italy	Comisana
KF302449	B	Europe	Italy	Sopravissana
KF302451	B	Europe	Italy	Appenninica
KF302446	A	Europe	Italy	Merinizzata Italiana
KF302445	A	Europe	Italy	Comisana
HE577847	A	Middle East	Israel	Afec-Assaf
KF302441	A	Europe	Italy	Comisana
KF302450	B	Europe	Italy	Appenninica
KF302443	A	Europe	Italy	Comisana
KF302440	A	Europe	Italy	Comisana
KF302444	A	Europe	Italy	Comisana
HM236183	E	Middle East	Turkey	Tuj
HM236182	E	Middle East	Israel	Awassi
HM236179	C	Middle East	Turkey	Morkaraman
HM236178	C	Middle East	Turkey	Karakas
KF312238	-	Europe	Cyprus	Ovis orientalis
HE577850	C	Middle East	Israel	Afec-Assaf

Table S2. The 45 published complete mitochondrial sequences from GeneBank used in the phylogenetic analysis.

Cyt b	Haplogroup	Continent	Country	Breed
D84203	B	No info	No info	Ovis musimon
DQ097407	B	Middle East	Turkey	Tuj
DQ097408	B	Middle East	Turkey	Karayaka
DQ097409	B	Middle East	Turkey	Hemshin
DQ097410	B	Middle East	Turkey	Hemshin
DQ097411	B	Middle East	Turkey	Tuj
DQ097412	B	Middle East	Turkey	Akkaraman
DQ097413	B	Middle East	Turkey	Tuj
DQ097414	B	Middle East	Turkey	Tuj
DQ097415	A	Middle East	Turkey	Akkaraman
DQ097416	A	Middle East	Turkey	Karayaka
DQ097417	A	Middle East	Turkey	Karayaka
DQ097418	A	Middle East	Turkey	Morkaraman
DQ097419	A	Middle East	Turkey	Morkaraman
DQ097420	A	Middle East	Turkey	Morkaraman
DQ097421	A	Middle East	Turkey	Morkaraman
DQ097422	A	Middle East	Turkey	Morkaraman
DQ097423	C	Middle East	Turkey	Akkaraman
DQ097424	C	Middle East	Turkey	Hemshin
DQ097425	C	Middle East	Turkey	Karayaka
DQ097426	C	Middle East	Turkey	Akkaraman
DQ097427	C	Middle East	Turkey	Akkaraman
DQ097428	C	Middle East	Turkey	Hemshin
DQ097429	C	Middle East	Turkey	Morkaraman
DQ097430	C	Middle East	Turkey	Karayaka
DQ309016	A	Asia	China	No info
DQ309017	C	Asia	China	No info
DQ309018	C	Asia	China	No info
DQ309019	B	Asia	China	No info
DQ309020	B	Asia	China	No info
DQ309021	A	Asia	China	No info
DQ320090	B	Europe	Scotland	Finn Dorset
DQ320091	B	Europe	Scotland	Finn Dorset
DQ903208	A	Asia	China	Bashibai
DQ903209	C	Asia	China	Bashibai
DQ903210	B	Asia	China	Bashibai
DQ903211	A	Asia	China	Ganjia

Cyt b	Haplogroup	Continent	Country	Breed
DQ903212	B	Asia	China	Ganjia
DQ903213	B	Asia	China	Henan
DQ903214	C	Asia	China	Henan
DQ903215	A	Asia	China	Henan
DQ903216	A	Asia	China	Hanzhong
DQ903217	B	Asia	China	Hanzhong
DQ903218	A	Asia	China	Lanzhou
DQ903219	A	Asia	China	Lanzhou
DQ903220	B	Asia	China	Lanzhou
DQ903221	B	Asia	China	Oula
DQ903222	B	Asia	China	Minshan black sheep
DQ903223	C	Asia	China	Minshan black sheep
DQ903224	B	Asia	China	Minshan black sheep
DQ903225	B	Asia	China	Skudde
DQ903226	B	Asia	China	Skudde
DQ903227	B	Asia	China	Tashikuergan
EU365977	B	Europe	France	Ovis musimon
EU365990	B	Europe	France	Ovis musimon
HE577847	A	Middle East	Israel	Afec-Assaf
HE577848	B	Middle East	Israel	Afec-Assaf
HE577849	B	Middle East	Israel	Afec-Assaf
HE577850	C	Middle East	Israel	Afec-Assaf
HM236174	A	Europe	Germany	Merino
HM236175	A	Europe	England	Romney
HM236176	B	Middle East	Turkey	Karakas
HM236177	B	Middle East	Turkey	Karakas
HM236178	C	Middle East	Turkey	Karakas
HM236179	C	Middle East	Turkey	Morkaraman
HM236180	D	Middle East	Turkey	Morkaraman
HM236181	D	Middle East	Turkey	Morkaraman
HM236182	E	Middle East	Israel	Awassi
HM236183	E	Middle East	Turkey	Tuj
HM236184	B	Europe	Germany	Mouflon
JX567772	A	Asia	China	Tibetan
JX567774	C	Asia	China	Tibetan
JX567775	A	Asia	China	Tibetan
JX567776	A	Asia	China	Tibetan
JX567777	A	Asia	China	Tibetan

Cyt b	Haplogroup	Continent	Country	Breed
JX567778	A	Asia	China	Tibetan
JX567779	A	Asia	China	Tibetan
JX567780	B	Asia	China	Tibetan
JX567781	C	Asia	China	Tibetan
JX567782	A	Asia	China	Tibetan
JX567783	A	Asia	China	Tibetan
JX567784	C	Asia	China	Tibetan
JX567785	A	Asia	China	Tibetan
JX567786	B	Asia	China	Tibetan
JX567787	B	Asia	China	Tibetan
JX567788	B	Asia	China	Tibetan
JX567789	A	Asia	China	Tibetan
JX567790	A	Asia	China	Tibetan
JX567791	A	Asia	China	Tibetan
JX567792	C	Asia	China	Tibetan
JX567793	A	Asia	China	Tibetan
JX567794	A	Asia	China	Tibetan
JX567795	A	Asia	China	Tibetan
JX567796	B	Asia	China	Tibetan
JX567797	A	Asia	China	Tibetan
JX567798	C	Asia	China	Tibetan
JX567799	A	Asia	China	Tibetan
JX567800	C	Asia	China	Tibetan
JX567801	A	Asia	China	Tibetan
JX567802	A	Asia	China	Tibetan
JX567803	A	Asia	China	Tibetan
JX567804	A	Asia	China	Tibetan
JX567805	B	Asia	China	Tibetan
JX567806	C	Asia	China	Tibetan
JX567807	A	Asia	China	Tibetan
JX567808	A	Asia	China	Tibetan
JX567809	C	Asia	China	Tibetan
JX567810	C	Asia	China	Tibetan
JX567811	B	Asia	China	Tibetan
JX567812	C	Asia	China	Tibetan
JX567813	A	Asia	China	Tibetan
JX567814	A	Asia	China	Tibetan
JX567815	A	Asia	China	Tibetan

Cyt b	Haplogroup	Continent	Country	Breed
JX567816	A	Asia	China	Tibetan
JX567817	A	Asia	China	Tibetan
JX567818	A	Asia	China	Tibetan
JX567819	C	Asia	China	Tibetan
JX567820	A	Asia	China	Tibetan
JX567821	B	Asia	China	Tibetan
JX567822	A	Asia	China	Tibetan
JX567823	A	Asia	China	Tibetan
JX567824	A	Asia	China	Tibetan
JX567825	A	Asia	China	Tibetan
JX567826	B	Asia	China	Tibetan
JX567827	A	Asia	China	Tibetan
JX567828	B	Asia	China	Tibetan
JX567829	B	Asia	China	Tibetan
JX567830	A	Asia	China	Tibetan
JX567831	A	Asia	China	Tibetan
KF302440	A	Europe	Italy	Comisana
KF302441	A	Europe	Italy	Comisana
KF302442	A	Europe	Italy	Comisana
KF302443	A	Europe	Italy	Comisana
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KF302447	B	Europe	France	Lacaune
KF302448	B	Europe	Italy	Gentile di Puglia
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KF302454	B	Europe	Italy	Sopravissana
KF302455	B	Europe	Italy	Gentile di Puglia
KF302456	B	Europe	Italy	Sopravissana
KF302457	B	Europe	Italy	Gentile di Puglia
KF302458	B	Europe	Italy	Sopravissana
KF302459	B	Europe	Italy	Merinizzata Italiana
KF302460	B	Europe	France	Lacaune
KF302461	B	Europe	Italy	Comisana

Cyt b	Haplogroup	Continent	Country	Breed
KF302462	B	Europe	Italy	Comisana
KF312238	C	Europe	Cyprus	Mouflon
KF977845	B	No info	No info	Djallonke
KF977846	B	No info	No info	Sahelian
KF977847	A	No info	No info	Small-tailed Han
KJ954145	B	No info	No info	Texel
NC_001941	B	Europe	Germany	Merinolandschaf
JX567773	C	Asia	China	Tibetan

Table S3. The 159 published complete cytochrome b sequences from GeneBank used in the phylogenetic analysis.